AD_____

GRANT NUMBER: DAMD17-94-J-4081

TITLE: Cyclin E, a Potential Prognostic Marker in Breast Cancer

PRINCIPAL INVESTIGATOR: Khandan Keyomarsi, Ph.D.

CONTRACTING ORGANIZATION: Health Research, Inc.
New York State Department of Health
Albany, New York 12209-1718

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 1 Oct 94 - 30 Sep 95

4. TITLE AND SUBTITLE
Cyclin E, a Potential Marker in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4081

6. AUTHOR(S)
Khandan Keyomarsi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Health Research, Inc.
New York State Department of Health
Albany, New York 12209-1718

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We have shown severe quantitative and qualitative alterations in cyclin E protein expression independent of the S-phase fraction in human mammary epithelial cell lines as well as surgical material obtained from patients with various malignancies. In addition in breast cancer, the alterations in cyclin E expression become progressively worse with increasing stage and grade of the tumor suggesting its potential use as a new prognostic marker. We also analyzed the kinase activity associated with cyclin E in a number of asynchronous normal and tumor cell lines as well as synchronized population of these cells. These analyses revealed that cyclin E associated kinase activity is present at much higher levels in all tumor versus normal cell lines and that while in normal cells cyclin E activity is cell cycle regulated, in tumor cells it remains as an active complex throughout the cell cycle. To analyze cyclin E for possible alterations which could result in its constitutive expression, we reverse transcribed and polymerase chain reaction (RT-PCR) amplified cyclin E cDNA from a tumor line (MDA-MB-157), cloned and sequenced the products and found two novel truncations in the cyclin E coding region. These truncations were found to be of general occurrence in most normal and tumor cell lines as well as normal adjacent and tumor tissue samples from breast cancer patients as detected in RT-PCR assays. However Western blot analysis indicated that the multiple isoforms of cyclin E protein were expressed only in the tumor tissue samples.Collectively these observation suggest the presence of more than one form of cyclin E cDNA in all cells, normal and tumor, and once translated in tumor cells, the protein product of these truncated forms may give rise to a constituitively active form of cyclin E, capable of phosphorylating substrates at all points in the tumor cell cycle.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Cell cycle, cyclin E, breast cancer, prognostic marker	44
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

DOD - See DoDD 5230.24, "Distribution Statements on Technical Documents."
DOE - See authorities.
NASA - See Handbook NHB 2200.2.
NTIS - Leave blank.

Block 12b. Distribution Code.

DOD - Leave blank.
DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
NASA - Leave blank.
NTIS - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

K.K. Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

K.K. Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

K.K. For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

K.K. In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

K.K. In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

KK In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

K. Keyman Vice 10/24/95

PI - Signature Date

TABLE OF CONTENTS

5. Introduction:

The overall purpose of this study is to use the altered expression of cyclin E as a diagnostic/prognostic marker and to investigate the mechanisms and repercussions of this alteration in breast cancer.

Cyclins are prime cell cycle regulators and central to the control of cell proliferation in eukaryotic cells via their association with and activation of cyclin-dependent protein kinases 1-7 (cdks) (reviewed in [1-6]. Cyclins were first identified in marine invertebrates as a result of their dramatic cell cycle expression patterns during meiotic and early mitotic divisions [7-10]. Several classes of cyclins have been described and are currently designated as cyclins A-H, some with multiple members. Cyclins can be distinguished on the basis of conserved sequence motifs, patterns of appearance and apparent functional roles during specific phases and regulatory points of the cell cycle in a variety of species. The cdk partners of several of these cyclins have also been identified: Cyclin A forms a complex with cdc2 (cdk1) and cdk2, and is required both at mitosis and DNA replication [11-14]; cyclin B forms a complex solely with cdc2 and is required for entry into mitosis, (reviewed in [3]; cyclin D1, a cyclin active in the G1 phase of the cell cycle, forms complexes primarily with cdk4 and cdk6, while cyclin E, another G1 type cyclin, forms a complex with only cdk2 [3, 4, 15-19]. Lastly, cyclin H has been shown to form a complex with cdk7 and, together, they comprise the cdk-activating kinase (CAK) protein complex which activates the nascent cyclin/cdk complex via phosphorylation [20, 21]. Cyclin binding to a cdk enables the kinase to become active, initiating a complex kinase cascade that directs the cell into DNA synthesis and/or mitosis, reviewed in [22, 23].

The connection between cyclins and cancer has been substantiated with the D type cyclins [6, 9, 24]. Cyclin D1 was identified simultaneously by several laboratories using independent systems: It was identified in mouse macrophages due to its induction by colony stimulating factor 1 during

G1 [25]. It was also identified in complementation studies using yeast strains deficient in G1 cyclins [23, 26]; as the product of the bcl-1 oncogene [27], and as the PRAD1 proto-oncogene in some parathyroid tumors where its locus is overexpressed as a result of a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene [28-31]. In centrocytic B cell lymphomas cyclin D1 (PRAD1)/BCL1 is targeted by chromosomal translocations at the BCL1 breakpoint, t(11;14)(q13;q32) [32, 33]. Furthermore, the cyclin D1 locus undergoes gene amplification in mouse skin carcinogenesis, as well as in breast, esophageal, colorectal and squamous cell carcinomas [34-39]. Several groups have examined the ability of cyclin D1 to transform cells directly in culture with mixed results [9, 28, 40-46]. However, the overexpression of cyclin D1 was recently observed in mammary cells of transgenic mice and results in abnormal proliferation of these cells and the development of mammary adenocarcinomas [47]. This observation strengthens the hypothesis that the inappropriate expression of a G1 type cyclin may lead to loss of growth control.

Cyclins D2 and A have also been implicated in oncogenesis. The cyclin D2 gene appears to be the integration site of a murine leukemia provirus in mouse T cell leukemias, resulting in its overexpression [48]. Cyclin A was found to be the site of integration of a fragment of the hepatitis B virus genome in a hepatocellular carcinoma [49]. Cyclin A is also associated with the adenovirus transforming protein E1A in adenovirus transformed cells [50, 51].

Recently, we and others have reinforced the linkage between oncogenesis and the cell cycle by correlating the deranged expression of cyclins to the loss of growth control in breast cancer [35, 52]. Using proliferating normal versus human tumor breast cell lines in culture as a model system, we have described several changes that are seen in all or most of these lines. These include (i) an eight-fold amplification of the cyclin E gene in one tumor line, 64 fold overexpression of its mRNA, and altered expression of its protein, (ii) deranged expression of cyclin E protein in 10/10 tumor cell lines studied, (iii) increased cyclin mRNA stability, resulting in overexpression of

mitotic cyclins and cdc2 RNAs and proteins in 9/10 tumor lines, and iv) deranged order of appearance of cyclins in synchronized tumor cells, with mitotic cyclins appearing prior to G1 cyclins.

The most striking abnormality in cyclin expression we found, was that of cyclin E. Cyclin E protein not only was overexpressed in 10/10 breast tumor cell lines but it was also present in lower molecular weight isoforms than that found in normal cells [53]. We directly examined the relevance of cyclin derangement to *in vivo* conditions, by measuring the expression of cyclin E protein in tumor samples versus normal adjacent tissue obtained from patients with various malignancies [53]. These analyses revealed that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alteration in cyclin E protein expression independent of the S-phase fraction of the samples. In addition, the alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as PCNA or c-erb B2. These observations strongly suggested the potential use of cyclin E as a new prognostic marker.

During the first year of this application we have used cyclin E antibody as a prognostic marker for breast cancer by analyzing 400 more breast tumor tissue specimens for the alterations in cyclin E protein. We also compared the expression of cyclin E to expression of other tumor markers such as cyclin D1 and erbB2. We also have obtained information on the steroid receptor status as well as DNA ploidy and proliferation rate of each tumor tissue sample. We will compare these parameters to the stage of each cancer.

We have also begun to characterize the mechanisms of alteration of cyclin E using cultured cells. We show that the alteration of cyclin E protein levels is accompanied by very high H1 kinase activity which is constituitively active throughout the cell cycle in tumor but not in normal mammary breast epithelial cells. Sequence analysis of cyclin E cDNAs amplified from tumor cells

revealed the presence of two different, deletional variants of cyclin E sequence. Interestingly, these two variant transcripts of cyclin E were also present in most normal and tumor cell lines as well as most normal adjacent and tumor tissue samples examined. We also show that these cyclin E variants are biochemically active in vitro.

6: Body (Results)

During the first year of this grant application we performed two studies as outlined in our grant application's Specific Aims 1 and 2. The first study consisted of using cyclin E antibody as a diagnostic/prognostic marker for breast cancer which as outlined in the first aim of our application will take 3 years to complete. During the first year we have collected 400 tumor tissue samples from breast cancer patients diagnosed with different stages of breast cancer ranging from pre-malignant to highly invasive. We have extracted RNA, DNA and protein from most of these samples. Due to limited sample size received for each patient (i.e 0.1-0.2 g of tissue), protein was initially extracted from all samples and if there was tumor sample left over, DNA and RNA were also extracted. The protein extracts from these 300 samples were then subjected to Western blot analysis and the expression of cyclin E was compared and correlated with other known prognostic markers examined in the same samples. The prognostic markers include, cyclin D1, erbB-2, as well as PCNA to determine the proliferative activity of these samples. We also have information on the estrogen and progesterone receptor status of each sample as well as ploidy and proliferation rate as measured by KI-67. We are currently in the process of performing immunohistochemistry with the antibodies we used for Western blot analysis. We have also begun to correlate the extent of cyclin E alteration to the stage of the breast cancer samples examined. As this is an ongoing study we anticipate to conclude these studies by the end of the third year of this application, at which time the results of our final analysis will be submitted.

The second study involved the utilization of cyclin E deletional mutations to detect early metastatic breast cancer. The first task of this study consisted of screening breast cancer cell lines for the

presence /generality of cyclin E mutations using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. We then extended these studies to RNA extracted form breast tissue samples to determine if cyclin E mutations also occur in the in vivo conditions. These analysis are summarized in figures 3-6 of this report and demonstrate a very intriguing finding. The cyclin E forms we had referred to as deletional mutations turned out not to be deletional mutations, but rather splicing variants of cyclin E that are present in both normal and tumor cell lines and tissue samples. As discussed in the conclusion, we believe that these truncated forms of cyclin E will help us decipher the mechanisms of alteration of cyclin E in breast cancer. In fact we have initiated our studies on elucidating the mechanism of alteration of cyclin E which is part of the third Specific Aim of our application by investigating the cell cycle expression of cyclin E in normal versus tumor cells at the level of protein kinase activities as shown in Figure 1 and 2 of this reports. Even though these studies were intended for the second year of this application we initiated some of the experiments during the first year, and the results are quite interesting.

Elevated Cyclin E associated kinase activity in breast cancer cells.

To test the hypothesis that the altered expression pattern of cyclin E protein found in tumor cell lines and tissue samples [53] is associated with increased cyclin E kinase activity, we compared cyclin E expression and activity in two normal versus five breast cancer cell lines (Fig 1). The two normal cell lines are the normal cell strain, 76N (Fig 1, lane 1), obtained from reduction mammoplasty and a near diploid immortalized cell line MCF-10A (Fig 1, lane 2) [54]. 76N is a mortal cell strain since it rapidly proliferates (doubling time of 24-27 hrs) for multiple passages before senescence at around passage 20 [55]. The MCF-10A cell line is a spontaneously immortalized human breast epithelial cell line which can be cultured indefinitely. This cell line has no tumorigenicity potential but retains characteristics of a normal breast epithelial cell line [54].

We examined the pattern of cyclin E protein expression in normal versus tumor cell lines using monoclonal and polyclonal antibodies to cyclin E on Western blots (Fig 1A). Similar immunoblot

banding patterns were obtained with either the monoclonal or polyclonal antibody to cyclin E, confirming the specificity of the multiple bands. However, the patterns of cyclin E protein expression was different between normal and tumor cells. Both cyclin E antibodies recognized one major protein migrating at ~50KDa, and two much less abundant lower molecular weight forms, in the two normal cell lysates. In the tumor cell lysates on the other hand, the same antibodies recognized three (lane 3), two (lanes 5-7) or one (lane 4) additional and highly abundant isoforms of cyclin E protein that in each case revealed a different pattern from that of the normal cells.

We next analyzed the cyclin E associated protein kinase activity in all cells by measuring the phosphorylation of histone H1 in immunoprecipitates made with the polyclonal antibody to cyclin E (Fig 1B). In all of the tumor cell lysate immunoprecipitates, the activity levels of cyclin E-associated kinase were significantly higher than that of both normal cells. For example, in MDA-MB-436 and SKBR3 tumor cell lines (lanes 4 and 6) which express only the lower molecular weight isoforms of cyclin E protein, the associated kinase activity was six fold greater than that of the normal cells which express mainly the high molecular weight, 50KDa, form of cyclin E protein. Similarly, the other tumor lines containing altered patterns of cyclin E expression, had significantly higher cyclin E-associated H1-kinase activity as compared to the normal cell strains.

Lack of cell cycle regulation of cyclin E in breast cancer cells.

In one tumor line, MDA-MB-157 (lane 3), the level as well as the associated kinase activity of cyclin E protein was the highest of all the tumor cell lines examined. Previous studies [52] showed that this overexpression is in part due to an 8-fold amplification of the cyclin E gene and 64 fold overexpression of its mRNA in this cell line. The cyclin E gene is amplified in tandem and is not associated with gross genomic rearrangements (data not shown). To investigate whether the signals required for normal regulation of cyclin E expression are altered or lost in tumor cells, the cell cycle expression of cyclin E protein and its associated kinase activities in the MDA-MB-157 cell line were compared to normal mammary epithelial 76N cells (Fig 2).

Both cell lines were synchronized in the G1/S border by double thymidine block. Synchrony of both cell types at several times after release from the block was monitored by flow cytometry[1] (Fig 2D). At various times after release from treatment for synchronization, cells were harvested and extracted proteins were analyzed on Western blots with antibodies to cyclins E and A (Fig 2A). In normal 76N cells, the pattern of expression of cyclin E and cyclin A proteins is consistent with that seen for other normal cell types with levels rising prior to S phase and oscillating thereafter in the cell cycle (Koff *et al.*, 1992). In addition there is only one major form (i.e., 50KDa) of cyclin E protein detected and there is a shift in the timing of when cyclin E versus cyclin A appears in the cell cycle of these normal epithelial cells. However, in the tumor cells, cyclin E protein does not appear to be cell cycle regulated and multiple isoforms of the protein are also present with similar signal intensities and banding patterns during the time intervals examined. In the same tumor cell extracts, cyclin A protein is cell cycle regulated with peak levels coinciding with peak S and early M phase. Hence, it appears that in this tumor cell line, cyclin E is abnormally regulated during the cell cycle.

In order to compare the kinase activity associated with cyclin E and cdk2 in normal and tumor cells, we measured the phosphorylation of histone H1 in immunoprecipitates prepared from synchronous cell extracts using antibody to either cyclin E or cdk2 (Fig 2B). There were two significant differences found between normal and tumor cells: First, in the length of time which an active cyclin E/cdk2 complex is present, and secondly in the amount of kinase activity associated with cyclin E versus cdk2 during the normal and tumor cell cycles. In normal cells, both cyclin E associated kinase and cdk2 activities are cell cycle regulated, coinciding with the levels of cyclins E

---

[1]Although the doubling times of the normal 76N and tumor MDA-MB-157 cells are slightly different (27 and 36 h, respectively), their flow cytometry profiles are similar, indicating equal DNA content distribution in different cell cycle phases (data not shown).

and A protein expression (Fig 2A). In addition, the cdk2 activity is one order of magnitude (i.e 10 fold) higher than cyclin E associated activity, consistent with cdk2's ability to form an active complex with other cyclins besides cyclin E in normal cells (Fig. 2B). Hence, cyclin E in these normal cells is indeed cell cycle regulated and the signals required for such regulation are intact both at the protein expression level and kinase activity.

In tumor cells, on the other hand, cyclin E is not cell cycle regulated and remains in a catalytically active complex throughout the cell cycle resulting in a constitutive pattern of histone H1 phosphorylation. The basal levels of cyclin E associated kinase activity during the tumor cell cycle, at any time interval examined, are at least 20 times higher than that of the normal cells (Fig 2C). Cdk2, a kinase which binds to both cyclin E and A, is also constituitively active during the cell cycle. However, cdk2 activity in this tumor cell line is only 2 fold higher than cyclin E associated kinase activity, presumably due to the abundance of cyclin E protein which is capable of sequestering cdk2. When cyclin A protein levels are induced in the tumor cells, there is only a 30% additional induction in cdk2 associated activity. These observations suggest that cyclin E protein, which is constituitively expressed in the cell cycle of tumor cells, also results in an active kinase complex throughout the cell cycle. Furthermore, since the same cyclin-dependent kinase can be regulated by both cyclins E and A, increased levels of cyclin E may overcompensate for cyclin A regulation, again resulting in a constituitively active and abundant cyclin E/cdk2 complex.

Isolation of variant forms of cyclin E transcripts.

In an attempt to determine the presence of any potential alterations in the cyclin E gene in MDA-MB-157, we amplified the entire cyclin E coding region of this cell line by reverse transcription-polymerase chain reaction amplification (RT-PCR), cloned these products and analyzed their DNA sequence (Fig 3). Using a pair of primers flanking the coding sequence of cyclin E gene, we observed at least two distinct PCR products ranging in size from 1.0 to 1.2 kb from the MDA-MB-157 RT template (Fig 3A). The product from the control (cyclin E plasmid DNA) was of 1.2 kb,

corresponding to the full length cyclin E cDNA isolated from a HeLa cDNA library (Koff *et al.*, 1991; Lew *et al.*, 1991). We cloned the RT-PCR products from the MDA-MB-157 cell line and confirmed their identity by Southern blotting and by DNA sequencing (data not shown). Three independent RT-PCR reactions were performed on freshly isolated RNA from this cell line. Fifteen clones from each RT-PCR reaction were examined further. Sequence analyses revealed two types of deletional variants of the cyclin E gene, as well as an unequivocally normal sequence, from the MDA-MB-157 cell line (Fig 3).

The PCR products containing these two deletional variants were termed cyclin E-Δ9 and cyclin E-Δ148 (Fig 3B). The deletion in clone cyclin E-Δ9 is a 9 base pair in-frame deletion of nucleotides 67-75 at the 5' end of the gene, while the deletion in clone cyclin E-Δ148 is a 148 base pair deletion of nucleotides 1000-1147 at the 3' end of the gene resulting in a frame shift transcript. Curiously, the 148 bp deletion in cyclin E Δ148 clone disrupts the PEST sequence motif of the gene, which is thought to be important for its role in degradation of the protein product [Koff, 1991 #2499; Lew, 1991 #2856]. The relative positions of these two newly identified deletions to the wild type sequence of cyclin E are shown in Fig 3B. We performed <u>in vitro</u> translation studies on these clones using T7 RNA polymerase (Fig 3C, D). RNA was translated in the presence of ^{35}S-methionine using a rabbit reticulocyte lysate, analyzed by SDS-PAGE and visualized by autoradiography. Cyclin E-wt and cyclin E-Δ9 protein products showed very similar electrophoretic mobilities (Fig 3C). [On a sequencing length SDS-PAGE, gel however, we were able to detect the slight (3 amino acids) molecular weight difference between the two clones, (data not shown)]. Cyclin E-Δ148 gives rise to a protein product which is ~5kd smaller than the cyclin E-wt, which would correspond to the loss of the 50 amino acids. To confirm that the protein products from <u>in vitro</u> translation reactions were indeed cyclin E, the cDNAs of the three different clones were transcribed and translated in the presence of unlabeled methionine and the products were subjected to Western blot analysis (Fig 3D). The protein products from cyclin-wt, Δ9 and Δ148 clones reacted strongly with the polyclonal antibody to cyclin E, suggesting that the <u>in vitro</u>

translated products of these clones are truncated forms of cyclin E. Interestingly, all the clones gave rise to two major protein products, migrating at ~45 and ~38kd for cyclin E-wt and Δ9 clones, and ~40 and ~33kd for cyclin E-Δ148 clone. It is not clear at this point whether the lower molecular weight protein product is a result of proteolytic cleavage, or result of translation initiation from a methionine site further downstream in the coding region.

Expression of cyclin E deletional variants in normal versus tumor cells and tissue samples.

Since these two altered forms of cyclin E cDNA were isolated from one tumor-derived cell line, we investigated the generality of expression of the cyclin E variants in a panel of 13 breast epithelial cell lines (Fig 4A). These cell lines included 3 normal mortal cell strains (lanes 1-3), 1 normal immortalized cell line (lane 4), and 9 tumor-derived breast cell lines (lanes 5-13). These analyses revealed the presence of multiple transcripts of cyclin E in all cell lines examined. However, no distinct differences were observed in their pattern of expression between normal versus tumor cell lines. Furthermore, sequence analysis of a cloned RT-PCR product of 76N normal cells revealed that the major transcript found in this normal cell strain is the Δ148 variant of cyclin E previously identified in MDA-MB-157 tumor cell line (data not shown). These observations indicate that the Δ148 RNA is expressed in all cells examined at an apparently higher level than the wild-type species of cyclin E RNA. To examine the specific expression of Δ9 and Δ148 in each cell line, we performed RT-PCR using primers that spanned the deleted sequences, such that only those cell lines containing cyclin E transcripts harboring these deletions would give rise to products. These analyses show that the Δ9 variant form of cyclin E is abundantly present in 3 cell lines, two of which are normal cell strains and one is the MDA-MB-157, the original cell line this variant form was isolated from (Fig 4B). In addition we find that the Δ148 is present in all cell lines examined (Fig 4C), confirming our previous observation that this variant form of cyclin E is the major transcript found in these cells (Fig 4A).

In order to apply our findings from culture studies to the in vivo condition, we examined whether the variant cyclin E transcripts were also expressed in tumor tissue specimens. We performed RT-PCR using RNA isolated from seven paired samples of human breast carcinoma and normal adjacent tissue (NAT) which are presented according to increased clinical stage (Fig 5). For this experiment, we used primers flanking the entire coding region of cyclin E in order to detect all variants of cyclin E which could contain deletions in the coding region. The RT-PCR products from NAT and tumor tissue samples ranged in size from 1.0 to 1.2 kb (Fig 5A), which are consistent with products obtained with cultured breast cells (Fig 4). Surprisingly, we found that not only did both NAT and tumor tissue samples express similar RT-PCR products corresponding to the cyclin E variants, but that no distinct difference could be found among paired samples as the clinical stage of the disease increases. On the other hand, when we subjected whole cell lysates prepared from these tissue specimens to Western blot analysis, we did observe cyclin E protein alterations which increased qualitatively and quantitatively as the stage of the disease increased. In high staged tumor samples, an antibody to cyclin E reacted strongly with at least three overexpressed proteins ranging in size from 35 to 50 KDa, while in the NAT samples, one major protein of 50KDa was present at very low levels, consistent with our previous observations [53]. Collectively these observations suggest that at the level of RNA there are no apparent differences between normal and tumor cells or between tissue samples in their ability to express the alternate transcripts of cyclin E. However, the alteration in cyclin E protein observed exclusively in tumor cells, likely occurs post transcriptionally or translationally to result in various forms of the protein detected in tumor but not normal cells or tissues.

Cyclin E deletional variants form biochemically active complexes with cdk2.

Based on the evidence that multiple cyclin E transcripts (Fig 4,5) are found in normal and tumor cells as well as in tissue samples, and that there is an active cyclin E/cdk2 protein complex present throughout the cell cycle of the MDA-MB-157 cell line (Fig 2), we asked whether these alternate transcripts of cyclin E can give rise to a biochemically active product. To investigate this question,

we overexpressed cyclin E and cdk2 in insect cells using the baculovirus expression system (Fig 6). Insect cells were co-infected with the recombinant baculovirus containing cdk2 and either cyclin E-wildtype (cycE-wt), cyclin E-Δ9, (cycE-Δ9), or cyclin E Δ148 (cycE-Δ148) cDNAs (Fig 6). At the indicated times (i.e. days) following infection, cell extracts were collected, homogenized and subjected to Western blot and histone H1 kinase analysis. Western blot analysis shows that there were similar levels of expression of the three cyclin E variants and cdk2 in the infected sf9 cells within one day of infection and thereafter during the course of experiment (Fig 6A). H1 kinase analysis reveal that when the cyclin E-wt/cdk2 co-infected insect cell lysates were immunoprecipitated with an antibody to cdk2, the immunoprecipitates were capable of phosphorylating histone H1 within one day of infection and an active cyclin E/cdk2 complex persisted throughout the experiment (Fig 6B, lanes 1-4). In insect cells co-infected with the two deleted variants of cyclin E, similar results were obtained illustrating that the complex which cycEΔ9(Fig 6C, lanes 5-8) or cycEΔ148 (Fig6C, lanes 9-12) formed with cdk2 is also active and is capable of phosphorylating histone H1. However a lower degree of activation was found compared to that with the cyclin E wildtype complex. There was a 2 fold difference in the ability of the cycEΔ9/cdk2 or cycEΔ148/cdk2 to phosphorylate histone H1 when compared to cyclin E wt/cdk2. These <u>in vitro</u> analyses suggest that once the cyclin E variant transcripts are translated, the protein products can give rise to a functionally active cyclin E complex capable of phosphorylating substrates such as histone H1.

7: Conclusions/Discussion:

The first Aim of our studies, use of cyclin E antibody as a diagnostic prognostic marker for breast cancer is an ongoing one. However, we have surpassed our initial goal of collecting and extracting 150 tissue samples per year by increasing this number to 400 samples. We also have completed our analysis of cyclin E and other tumor markers by Western blot analysis and are in the process of performing immunohistochemistry on these antibodies. We are working toward completion this

aim by the third year of this grant application as originally anticipated and hence do not anticipate any major changes or problems.

The second Aim of the application deals with utilizing the deletional mutations of cyclin E to detect early metastatic breast cancer. As described in detail in the results section, we provide evidence that these truncated forms of cyclin E are not deletional mutations and are in fact splicing variants of cyclin E found in normal and tumor cells and tissue samples. These truncated forms of cyclin E however provide us with a valuable tool to elucidate the mechanism of alteration of cyclin E as discussed below. We have also initiated the third specific Aim of our grant application by deciphering the regulation of cyclin E i normal and tumor cells and have found while in normal cells cyclin E is regulated with peaks of expression and activity prior to S phase, in tumor cells cyclin E pattern of expression and activity is constitutive throughout the cell cycle suggesting that it has lost its checkpoint control for cyclin E. Our scope of this Specific Aim will be to further decipher the mechanism of deregulation of cyclin E in breast cancer. Below is a discussion of the results presented in this report:

In an attempt to understand the relationship between the cell cycle and cancer, many laboratories have investigated the role cyclin/cdk complexes play in cancer. While cyclins D and A have been implicated in tumorigenesis, the role of other cyclins have been elusive and limited mainly to observations. Cyclin E is an interesting case since it shows an altered pattern of expression in all breast cancer cell lines and tumor tissue samples we have examined to date [53]. The cyclin E alterations include overexpression of the authentic-sized protein as well as expression of lower molecular weight isoforms found in tumor cells or tissues. We set out to decipher the mechanism responsible for these alterations by initially correlating the activity of cyclin E/cdk2 complexes with the expression pattern and level of cyclin E protein, both in exponentially growing and synchronized population of normal versus tumor cells. We find that regardless of which combination of the cyclin E (50Kda) and its lower molecular weight isoforms are expressed in

these tumor cell lines, the associated kinase activity is much higher in tumor than normal cells. Furthermore, we find that in synchronized populations of tumor cells, cyclin E is present in altered forms throughout the cell cycle and the kinase activity associated with it, or with cdk2, is also constituitively active. In addition the abundant and constitutive expression of cyclin E in these tumor cells result in sequestering of cdk2 away from other cyclins, such as cyclin A. This suggests that, there is a cyclin E/cdk2 complex which is abundantly and uniformly active in the tumor but not the normal cell cycle.

In order to determine whether the multiple forms of the cyclin E protein detected in tumor cells originate from different transcripts of cyclin E RNA, we performed RT-PCR and found two different deletional variants of cyclin E (i.e Δ9 and Δ148) expressed in MDA-MB-157 tumor cell line. Further analysis revealed that the most intriguing feature of the Δ9 and Δ148 variant forms of cyclin E is that there is no distinct difference in their mRNA expression in normal versus tumor cells or tissue samples. In addition there is little correlation between expression of these cyclin E variants at the level of RNA versus protein. Yet, we show here that at the level protein in tumor cells, a) cyclin E isoforms ranging in size from 35 to 50KDa are abundantly expressed (Figs 1,2, and 5), and that b) these protein isoforms of cyclin E are not subject to cell cycle regulation and may constituitively interact with cdk2 resulting in an active complex (Fig 2). Lastly, c) we also show that once Δ9 and Δ148 transcripts of cyclin E are allowed to express their protein products, the resulting proteins can bind to cdk2 and form active complexes in vitro (Fig 6). Collectively, based on these observations we suggest that the multiple protein isoforms of cyclin E detected in tumor cells are a result of altered post-transcriptional and/or translational regulation of cyclin E mRNAs. Hence, there may be a translational fidelity that has been altered/lost in tumor cells, allowing for the translation of these deletional variants of cyclin E to occur and once translated, they can form active complexes with cdk2 throughout the cell cycle (see Figs 2 and 6). Alternatively, there may be post-translational modification of cyclin E which is also altered or lost in tumor cells. The presence of lower molecular weight protein isoforms of cyclin E, barely

detectable in normal cell lysates (Fig 1, lanes 1 and 2) could also suggest that these isoforms of cyclin E are in fact translated in normal cells as well, but they are rapidly degraded. In tumor cells, the protein turnover is much longer, and as a result we can readily detect lower molecular weight isoforms of cyclin E which are highly abundant and functionally active.

One possibility for the presence of multiple transcripts of cyclin E is due to alternative splicing. Precedent for alternative splicing of cyclin E was originally reported by Sewing et. al. where they identified a splice variant of cyclin E, termed cyclin Es [56]. Like cyclin Es, there is a strong possibility that both cyclin EΔ9 and cyclin EΔ148 reported here, are results of alternative splicing as we find potential splice donor and acceptor sites at the deleted junctions of each transcript. However, the cyclin Es variant differs from those we report here in that cyclin Es lacks 49 amino acids within the cyclin box, and is 90% less abundant than the wildtype cyclin E sequence. This form is unable to associate with cdk2, is inactive in histone Hl kinase assays, and is unable to rescue a triple CLN mutation of S. cerevisiae [56]. Unlike cyclin Es, neither the cyclin E Δ9 nor the Δ148 transcripts disrupts the cyclin box, the consensus region which confers activity by its association to a cdk [57]. As a result, both Δ9 and Δ148 variants of cyclin E retain the ability to functionally bind to cdk2 and phosphorylate histone H1 in insect cells (Fig 6). The ability of these novel variants of cyclin E to form an active complex with cdk2 has implications for their biological functions. The Δ148 variant has another interesting feature in that the PEST sequence important for its degradation has been disrupted by this 148 base pair deletion. It is possible that the deletion of a PEST sequence may have an effect on turnover of Δ148 cyclin E, allowing it to remain active for a longer duration than the wild type form.

The data presented here suggest that the mechanisms responsible for the presence of the multi iso-forms of cyclin E protein in tumor cells may be due to a number of factors, one of which is the altered post-transcriptional or translation regulation of the deletional variants of cyclin E. However a question can be raised whether these two novel variant forms of cyclin E attribute to the cancer

phenotype. We present data that these two variants are not a result of deletional mutations in the cyclin E gene as they are expressed in both normal and tumor cells as well as tissue samples. However, they are not readily detected in normal cells either due to their lack of translation or rapid degradation. There is evidence that when the wild type cyclin E is overexpressed in normal cells the length of G1 is decreased, but cells are not transformed [58, 59]. With the discovery of the cyclin E variants that may be translated in tumor but not normal cells, the oncogenecity of these cyclin E forms can now be directly deciphered. A second question that our data has raised, is whether the lower molecular weight isoforms of cyclin E detected mainly in tumor cells are the protein products of the cyclin EΔ9 and/or Δ149 variant transcripts of cyclin E. By identification of these two variants, we can now utilize them as molecular probes to identify their protein products in tumor cells and tissues. Identification of the multiple protein isoforms of cyclin E will give us insight as to the regulation of this protein, which when complexed with cdk2 is thought to be rate limiting for the G1/S transition during the mammalian cell cycle. With an active cyclin E/cdk2 complex, substrates may be phosphorylated at altered points in the cell cycle resulting in loss of checkpoint control during the progression of G1 to S in tumor cells.

8. References

1. Elledge, S.J. and M.R. Spottswood, *A new human p34 protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of Xenopus Eg1.* EMBO J, 1991. **10**: p. 2643-2659.

2. King, R.W., P.K. Jackson, and M.W. Kirschner, *Mitosis in transition.* Cell, 1994. **79**: p. 563-571.

3. Nurse, P., *Ordering S phase and M phase in the cell cycle.* Cell, 1994. **79**: p. 547-550.

4. Sherr, C.J., *G1 phase progression: cycling on cue.* Cell, 1994. **79**: p. 551-555.

5. Heichman, K.A. and J.M. Roberts, *Rules to replicate by.* Cell, 1994. **79**: p. 557-562.

6. Hunter, T. and J. Pines, *Cyclins and cancer II: cyclin D and cdk inhibitors come of age.* Cell, 1994. **79**: p. 573-582.

7. Standart, N., J. Minshull, J. Pines, and T. Hunt, *Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte.* Dev. Biol., 1987. **124**: p. 248-254.

8. Swenson, K.I., K.M. Farrell, and J.V. Ruderman, *The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in Xenopus oocytes.* Cell, 1986. **47**: p. 861-870.

9. Sherr, C.J., *Mammalian G1 cyclins.* Cell, 1993. **73**: p. 1059-1065.

10. Evans, T., E. Rosenthal, J. Youngblom, D. Kistel, and T. Hunt, *Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division.* Cell, 1983. **33**: p. 389-396.

11. Minshull, J., R. Golsteyn, C.S. Hill, and T. Hunt, *The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and of at different times in the cell cycle.* EMBO J, 1990. **9**: p. 2865-2875.

12. Draetta, G., F. Luca, J. Westendorf, l. Brizuela, J. Ruderman, and D. Beach, Cell, 1989. **56**: p. 829-838.

13. Tsai, L.-H., E. Harlow, and M. Meyerson, *Isolation of the human cdk 2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase.* Nature, 1991. **353**: p. 174-177.

14. Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta, *Cyclin A is required at two points in the human cell cycle.* EMBO J, 1992. **11**: p. 961-971.

15. Xiong, Y., T. Connolly, B. Futcher, and D. Beach, *Human D-type cyclin.* Cell, 1991. **65**: p. 691-699.

16. Baldin, V., J. Likas, M.J. Marcote, M. Pagano, J. Barteck, and G. Draetta, *Cyclin D1 is a nuclear protein required fro cell cycle progresion in G1.* Genes Dev., 1993. **7**: p. 812-821.

17. Meyerson, M. and E. Harlow, *Identification of G1 kinase activity for cdk6, a novel cyclin D partner.* Mol. Cell Biol., 1994. **14**: p. 2077-2086.

18. Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J.M. Roberts, *Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family.* Cell, 1991. **66**: p. 1217-1228.

19. Koff, A., A. Giordano, D. Desia, K. Yamashita, J.W. Harper, S.J. Elledge, T. Nishomoto, D.O. Morgan, R. Franza, and J.M. Roberts, *Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle.* Science, 1992. **257**: p. 1689-1694.

20. Matsuoka, M., J. Kato, R.P. Fisher, D.O. Morgan, and C.J. Sherr, *Activation of cyclin-dependent kinase-4 (CDK4) by mouse MO15-associated kinase.* Mol. Cell Biol., 1994. **78**: p. 713-724.

21. Fisher, R.P. and D.O. Morgan, *A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase.* Cell, 1994. **78**: p. 713-724.

22. Hunt, T., *Cyclins and their partners: from a simple idea to complicated reality.* Seminars in Cell Biology, 1991. **2**: p. 213-222.

23. Xiong, Y. and D. Beach, *Population explosion in the cyclin family.* Curr. Biol., 1991. **1**: p. 362-364.

24. Hunter, T. and J. Pines, *Cyclins and Cancer.* Cell, 1991. **66**: p. 1071-1074.

25. Matsushime, H., M.F. Roussel, R.A. Ashman, and C.J. Sherr, *Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle.* Cell, 1991. **65**: p. 701-713.

26. Lew, D.J., V. Dulic, and S.I. Reed, *Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast.* Cell, 1991. **66**: p. 1197-1206.

27. Withers, D., R. Harvey, J. Faust, O. Melnyk, K. Carey, and T. Meeker, *Chanracterization of a candidate bcl-1 gene.* Mol. Cell Biol., 1991. **11**: p. 4846-4853.

28. Quelle, D.E., R.A. Ashmun, S.A. Shurleff, J.-y. Kato, D. Bar-Sagi, M.F. Roussel, and C.J. Sherr, *Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts.* Genes & Dev., 1993. **7**: p. 1559-1571.

29. Motokura, T. and A. Arnold, *Cyclin D and oncogenesis.* Curr. Opin. Genet. & Devel., 1993. **3**: p. 5-10.

30. Motokura, T., T. Bloom, H.G. Kim, H. Jüppner, J.V. Ruderman, H.M. Kronenberg, and A. Arnold, *A BCL1-linked candidate oncogene which is rearranged in parathyroid tumors encodes a novel cyclin.* Nature, 1991. **350**: p. 512-515.

31. Matsushime, H., M.F. Roussel, and C.J. Sherr, *Novel Mammalian cyclins (CYL genes) expressed during G1.* Symposia on Quantitative Biology: The Cell Cycle, Vol. 56. 1991, Cold Spring Harbor Laboratory Press. 69-74.

32. Rosenberg, C.L., H.G. Kim, T.B. Shows, H.M. Kronenberg, and A. Arnold, *Rearangement and overexpression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors.* Oncogene, 1991. **6**: p. 449-453.

33. Rosenberg, C.L., E. Wong, E.M. Pety, A.E. Bale, Y. Tsujimoto, N.L. Harris, and A. Arnold, *PRAD1, acandidate BCL1 oncogene: mapping and expression in centrocytic lymphoma.* Proc. Natl. Acad. Sci USA, 1991. **88**: p. 9638-9642.

34. Bianchi, A.B., S.M. Fischer, A.I. Robles, E.M. Rinchik, and C.J. Conti, *Overexpression of cyclin D1 in mouse skin carcinogenesis.* Oncogene, 1993. **8**: p. 1127-1133.

35. Buckley, M.F., K.J.E. Sweeney, J.A. Hamilton, R.L. Sini, D.L. Manning, R.I. Nicholson, A. deFazio, C.K.W. Watts, E.A. Musgrove, and R.L. Sutherland, *Expression and amplification of cyclin genes in human breast cancer.* Oncogene, 1993. **8**: p. 2127-2133.

36. Lammie, G.A., V. Fantl, R. Smith, E. Shuuring, S. Brookes, R. Michalides, C. Dickson, A. Arnold, and G. Peters, *D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1.* Oncogene, 1991. **6**: p. 439-444.

37. Jiang, W., S.M. Kahn, N. Tomita, Y.-J. Zhang, S.-H. Lu, and B. Weinstein, *Amplification and expression of the human cyclin D gene in esophageal cancer.* Cancer Res., 1992. **52**: p. 2980-2983.

38. Leach, S.F., S.J. Elledge, C.J. Sherr, J.K.V. Willson, S. Markowitz, K.W. Kinzler, and B. Vogelstein, *Amplification of cyclin genes in colorectal carcinomas.* Cancer Res., 1993. **53**: p. 1986-1989.

39. Jiang, W., Y.-J. Zhang, S.M. Kahn, M.C. Hollstein, R.M. Santella, S.-H. Lu, C.C. Harris, R. Montesano, and I.B. Weinstein, *Altered expression of cyclin D1 and retinoblastoma genes in human esophageal cancer.* Proc. Natl. Acad. Sci., 1993. **90**: p. 9026-9030.

40. Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg, *Regulation of retinoblastoma protein functions by ectopic expression of human cyclins.* Cell, 1992. **70**: p. 993-1006.

41. Hinds, P.W., S.F. Dowdy, E.N. Eaton, A. Arnold, and R.A. Weinberg, *Function of a human cyclin gene as an oncogene.* Proc. Natl. Acad. Sci, 1994. **91**: p. 709-713.

42. Resnitzky, D., M. Gossen, H. Bujard, and S.I. Reed, *Accelaration o the G1/S phase transition by expression of cyclins D1 and E with an unducible system.* Mol. Cell. Biol., 1994. **14**: p. 1669-1679.

43. Rosenwald, I.B., A. Lazaris-Karatzas, N. Sonenberg, and E.V. Schmidt, Molec. Cell. Biol., 1993. **13**: p. 7358-7363.

44. Jiang, W., S.M. Kahn, P. Zhou, Y.-J. Zhang, A.M. Cacace, A.S. Infante, S. Doi, R.M. Santella, and I.B. Weinstein, *Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progresion and gene expression.* Oncogene, 1993. **8**: p. 3447-3457.

45. Lovec, H., A. Sewing, F.C. Lucibello, R. Müller, and T. Möröy, *Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras:link between cell cycle congrol and malignant transformation.* Oncogene, 1994. **9**: p. 323-326.

46. Musgrove, E.A., C.S.L. Lee, M.F. Buckley, and S. R.L., *Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle.* Proc. Natl. Acad. Sci., 1994. **91**: p. 8022-8026.

47. Wang, T.C., R.D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E.V. Schmidt, *Mammary hyperplasia and carcinma in MMTV-cyclin D1 transgenic mice.* Nature, 1994. **369**: p. 669-671.

48. Hanna, Z., M. Jankowski, P. Tremblay, X. Jiang, A. Milatovich, U. Francke, and P. Jolicoeur, *The vin-1 gene, identified by provirus insertional mutagenesis, is the cyclin D2.* Oncogene, 1993. **8**: p. 1661-1666.

49. Wang, J., X. Chenivesse, B. Henglein, and C. Bréchot, *Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma.* Nature, 1990. **343**: p. 555-557.

50. Giordano, A., P. Whyte, E. Harlow, B.R. Franza Jr.,, D. Beach, and G. Draetta, *A 60 kd cdc2-associated polypeptide complexes with the ElA protein in adenovirus-infected cells.* Cell, 1989. **58**: p. 981-990.

51. Pines, J. and T. Hunter, *Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B.* Nature, 1990. **346**: p. 760-763.

52. Keyomarsi, K. and A.B. Pardee, *Redundant cyclin overexpression and gene amplification in brest cancer cells.* Proc. Natl. Acad. Sci. USA, 1993. **90**: p. 1112-1116.

53. Keyomarsi, K., N. O'Leary, G. Molnar, E. Lees, H.J. Fingert, and A.B. Pardee, *Cyclin E, a Potential Prognostic Marker for Breast Cancer.* Cancer Res., 1994. **54**: p. 380-385.

54. Soule, H.D., T.M. Maloney, S.R. Wolman, W.D. Peterson Jr., R. Brenz, C.M. McGrath, J. Russo, R.J. Pauley, R.F. Jones, and S.C. Brooks, *Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10.* Cancer Research, 1990. **50**: p. 6075-6086.

55. Band, V. and R. Sager, *Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types.* Proc. Natl. Acad. Sci. USA, 1989. **86**: p. 1249-1253.

56. Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano, *Human cyclin E, a nuclear protein essential for the G1-to-S phase transition.* Mol. Cell. Biol., 1995. **15**: p. 2612-2624.

57. Lees, E., B. Faha, V. Dulic, S.I. Reed, and E. Harlow, *Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner.* Genes Dev., 1992. **6**: p. 1874-1855.

58. Ohtsubo, M. and J.M. Roberts, *Cyclin-dependent regulation of G1 in mammalian fibroblasts.* Science, 1993. **259**: p. 1908-1912.

59. Resnitzky, D., G. M., H. Bujard, and S.I. Reed, *Acceleration of the G1/S phase transition oby expression of cyclins D1 and e with an inducible system.* Mol. Cell Biol., 1994. **14**: p. 1669-1679.

9. Appendix

The figure and figure legendss referred to in the text are presented in this section.

We are also including a copy of a manuscript summarizing the results in this annual report.

Figure Legends:

Figure 1: Correlation of cyclin E protein(s) to cyclin E associated kinase activity

(A) Western blot analysis of cyclin E expression in normal vs. tumor cells using 2 different cyclin E antibodies. Whole cell lysates were extracted from the 7 cell lines, (100 µg of protein extract/lane), run on a 10% acrylamide gel, and blotted as described in Materials and Methods. Lane 1, 76N normal human mortal breast epithelial cell strain; lane 2, MCF-10A normal immortalized human breast epithelial cell line; (lanes 3-7 are all human breast cancer cell lines) lane 3, MDA-MB-157; lane 4, MDA-MB-436; lane 5, ZR75T; lane 6, SKBR3; lane 7, MCF-7. The 50 KDa arrowhead points to the cyclin E protein of the predicted size. The other arrowheads point to the additional cyclin E isoforms observed in the tumor cell lines ranging in molecular weight from 35 to 42 KDa. Molecular mass standards were used in each gel to estimate the position of each band. **(B)** Cyclin-E associated histone H1 kinase activity. Equal amounts of proteins from cell lysates were immunoprecipitated with anti-cyclin E coupled to protein A beads using histone H1 as substrate. The associated kinase activities were quantified by scintillation counting.

Figure 2: Expression of cyclin E in synchronized normal 76N and tumor MDA-MB-157 breast cells. Both cell types were synchronized by double thymidine block procedure (see Materials and Methods). At the indicated times following release from double thymidine block, cell lysates were prepared and subjected to **A**: Western blot and **B**: Histone H1 kinase analysis. Protein (50µg) for each time point was applied to each lane of a 10% acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity purified polyclonal antibodies. The blots were stripped between the two assays in 100 mM ß-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8), and 2 % SDS for 30 min at 55° C. For Histone

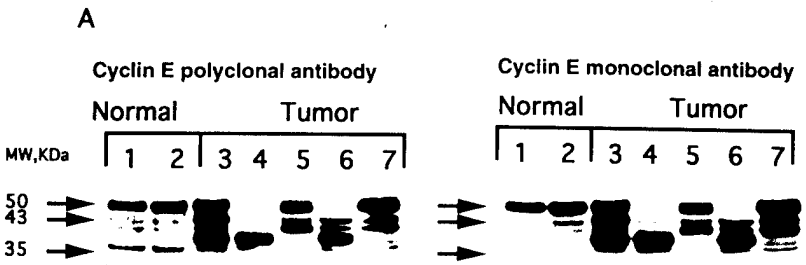
H1 kinase activity, equal amount of proteins (600 ug) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cyclin E (polyclonal) or anti-CDK2 (polyclonal) coupled to protein A beads using histone H1 as substrate. Panel B is the autoradiogram of the histone H1 SDS-PAGE gel and panel **C** shows the quantification of the histone H1 associated kinase activities by scintillation counting. Open symbols correspond to cyclin E associated kinase activity and closed symbols correspond to cdk2 activity. **D**: The relative percentage of cells in different phases of the cell cycle for each cell line at various times after release from double thymidine block was calculated from flow cytometric measurements of DNA content. (◆) cells in S phase, (O) cells in G2/M phase, and (❑) cells in G1 phase.

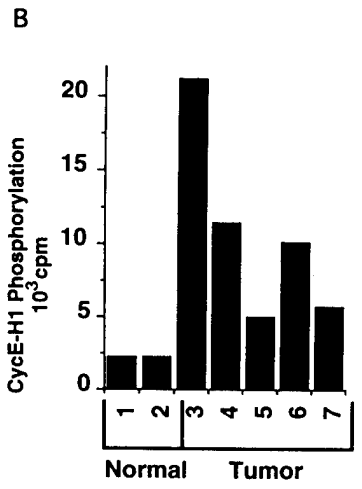
Figure 3: Identification and in vitro translation of cyclin E deletional transcripts. **A:** PCR amplified cyclin E coding sequence using primers (L1CYCE and R1CYLE) flanking the entire coding region of cyclin E. Lane 1: Molecular weight standards; lane 2: control template DNA, a plasmid containing a wild type cyclin E coding sequence; Lane 3: RT-PCR amplification of cyclin E using RNA from MDA-MB-157; lane 4: Molecular weight standards. PCR conditions were carried out as described in Materials and Methods. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Molecular weight markers in base pairs are indicated (left). **B:** Relative position of cyclin E Δ9 and Δ148 deletions to the wildtype cyclin E sequence. The two arrows flanking the cyclin E coding region refer to the position of R1 (i.e., R1CYCE) and L1 (i.e., L1CYCE) oligonucleotides used for the RT-PCR reactions. **C:** The cDNAs of cyclin E clones were subcloned into PCR II vector and transcribed and translated in vitro using T7 RNA polymerase-rabbit reticulocyte lysate system in the presence of ^{35}S-methionine and products were analyzed on a 10% SDS-PAGE followed by autoradiography. **D:** The in vitro translated cyclin E protein products from the three different clones (in the absence of radioactivity) were subjected to Western blot analysis and hybridized to a polyclonal antibody to cyclin E.

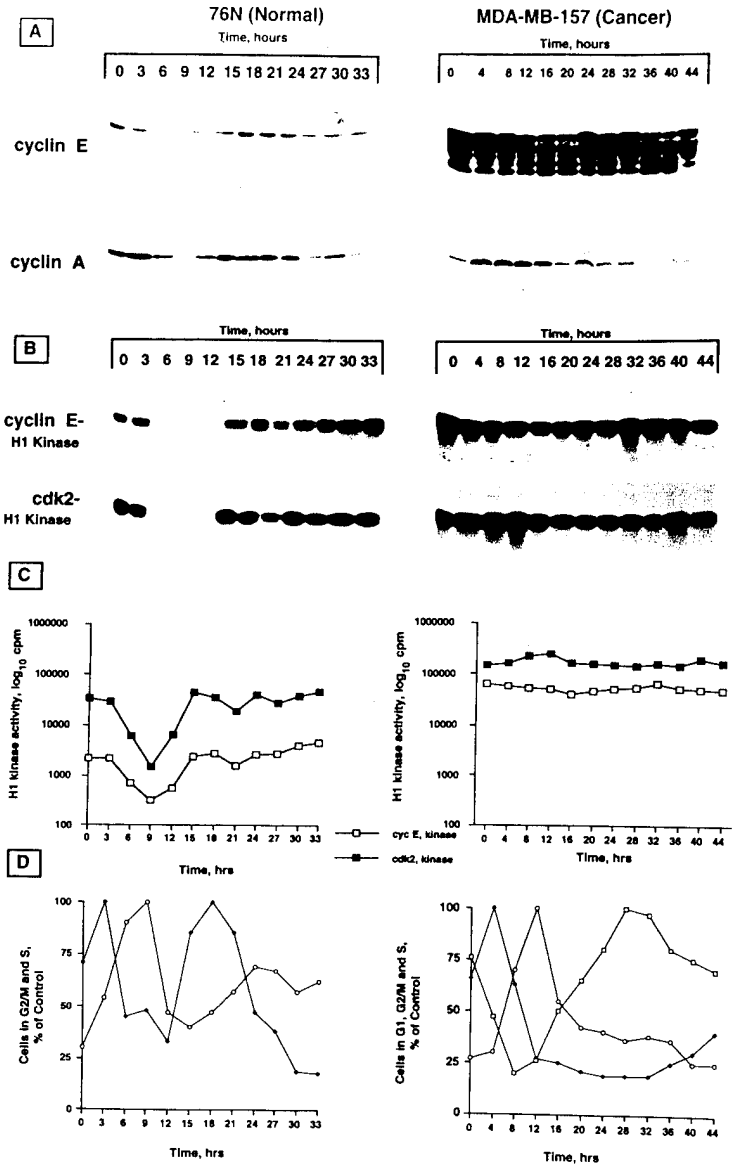
Figure 4: RT-PCR amplification of cyclin E Δ9 and Δ148 in normal and tumor-derived breast epithelial cell lines. RT-PCR amplification of cyclin E coding sequence from normal and tumor-derived breast epithelial cell lines using **A**: primers (L1CYCE and R1CYCE) flanking the coding region of cyclin E and amplifying wildtype cyclin E sequences, as well as those containing internal deletions, **B**: primers (LMEMARK3 and R1CYCE) spanning the Δ9 deletion and amplifying only those cyclin E sequences harboring the Δ9 internal deletion of cyclin E and, **C**: primers (L1CYCE and RMEMARK3) spanning the Δ148 internal deletion and amplifying only those cyclin E sequences containing the Δ148 deletion. The cell lines used are as follows: Lane 1, 70N; lane 2, 81N; lane 3, 76N; lane 4, MCF-10A ; lane 5, MCF-7; lane 6, MDA-MB-157; lane 7, MDA-MB-231; lane 8, MDA-MB-436; lane 9, T47D; lane 10, BT-20T; lane 11, HBL-100; lane 12, Hs578T, and lane 13, ZR75T. Normal cells are represented in lanes 1-4 and tumor-derived cell lines in lanes 5-13; M, Molecular weight size markers.

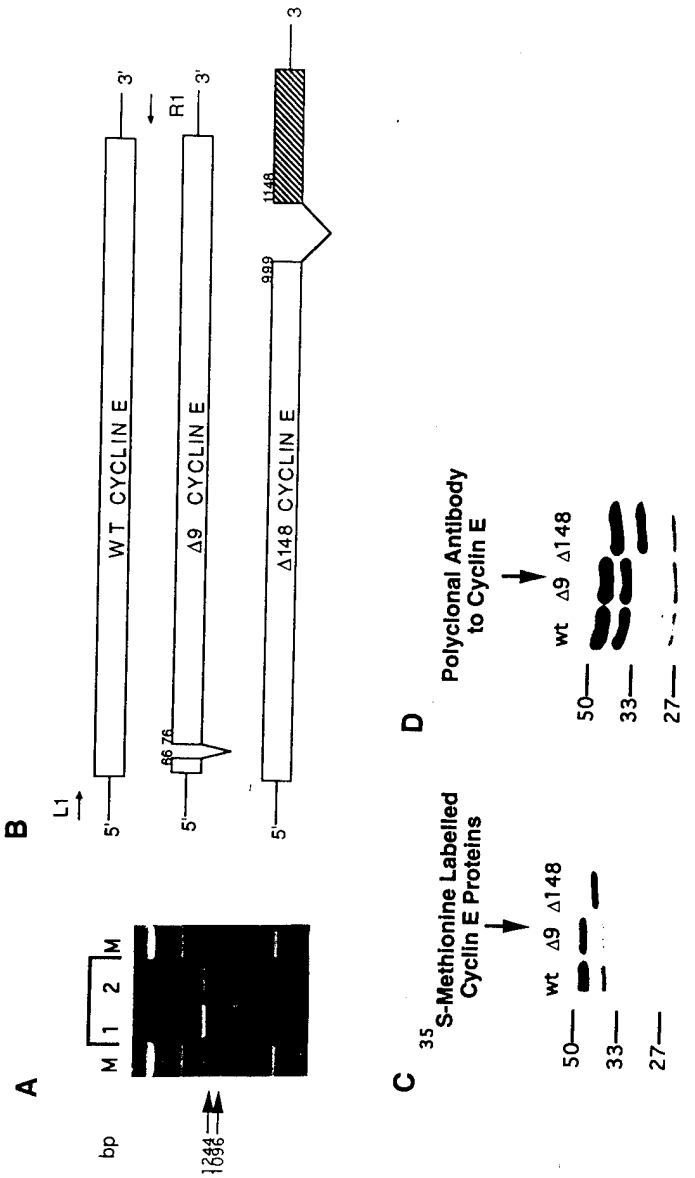
Figure 5: Comparison of RT-PCR amplified products of cyclin E with their expression in breast cancer specimens. **A**: RT-PCR amplification of cyclin E coding sequence using total RNA isolated from seven pairs of normal adjacent (NAT) and tumor tissue samples with primers (L1CYCE and R1CYCE) flanking the coding region of cyclin E as described in figure 4A. **B**: Western blot analysis of whole cell lysates (100 μg) were prepared from NAT and tumor tissue specimens and probed with a monoclonal antibody to cyclin E. Breast cancer types and histological/tumor grades are as follows: Lanes 1-2, intraductal carcinoma of the breast, Stage T1,N0,M0, Grade I; Lanes 3-4, invasive well differentiated ductal carcinoma, Stage T1,N0,M0, Grade I; Lanes 5-6, intraductal carcinoma, Stage T1/2,N0,M0, Grade I; Lanes 7-8, invasive and intraductal carcinoma, Stage T2,N0,M0, Grade II; Lanes 9-10, in situ and infiltrating ductal carcinoma, Stage T2,N1,M0, Grade II/III; Lanes 11-12, infiltrating ductal carcinoma, Stage T3,N0,M0, Grade II/III; Lanes 13-14, invasive ductal carcinoma, Stage 4,N0,M0, Grade III. Molecular mass standards were used on each gel to estimate the position of each band.

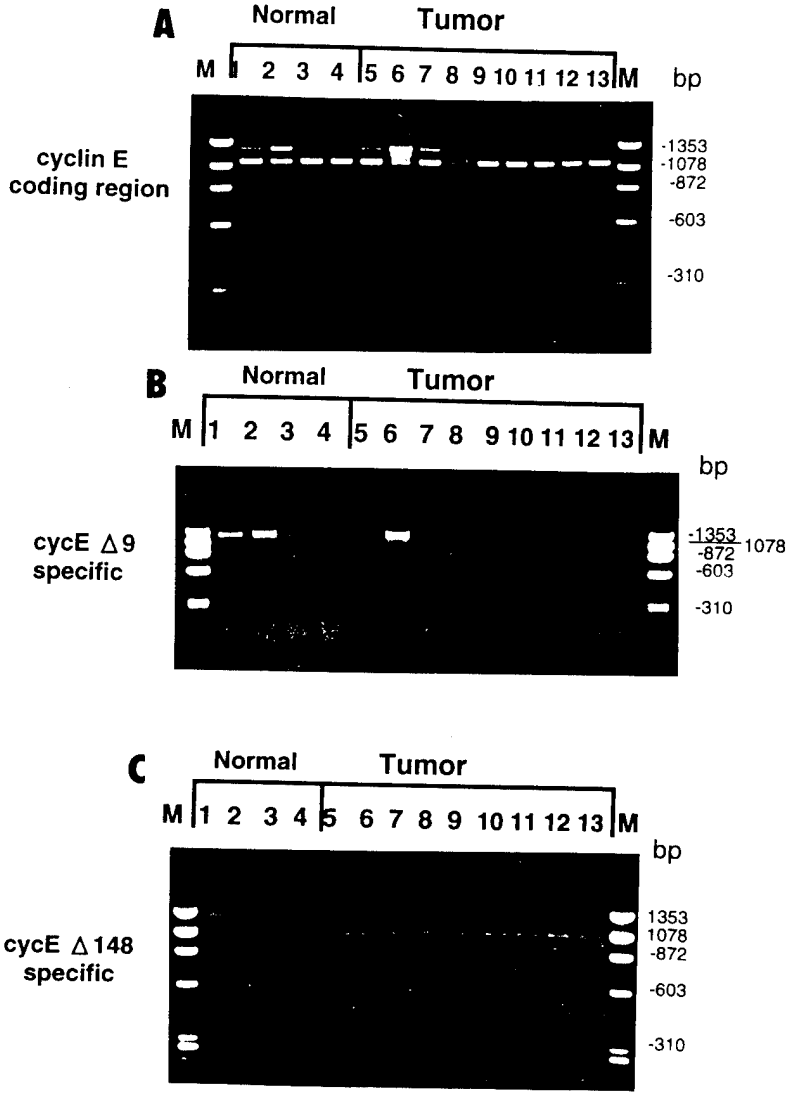
Figure 6: Activation of cdk2 by cyclin E wildtype and its deletional variants in insect cells. Cell lysates were prepared from insect cells co-infected with baculovirus containing the different cyclin E constructs and cdk2 at the indicated time intervals (days) following co-infection. **A**: Equal amounts (50 ug) of protein were added to each lane; the gel was then subjected to Western blot analysis with polyclonal antibody to cyclin E or to cdk2. C: Control lane corresponding to 50 ug of extracts from insect cells infected with either cyclin E wt alone, or cdk2 alone baculovirus. **B:** Histone H1 kinase assays were also performed on the same cell extracts by immunoprecipitating equal amount of cell lysate with polyclonal antibody to cdk2 coupled to protein A beads using histone H1 as substrate. The autoradiogram of the histone H1 SDS-PAGE gel is depicted. NRS: A control immunoprecipitate performed with Normal Rabbit Serum in place of cdk2 antibody.


A
Cyclin E polyclonal antibody
Normal
Tumor
MW,KDa
1 2 3 4 5 6 7
50
43
35
Cyclin E monoclonal antibody
Normal
Tumor
1 2 3 4 5 6 7

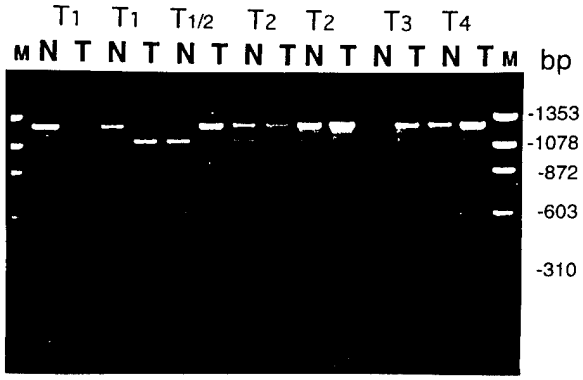

B
CycE-H1 Phosphorylation
10³cpm
20
15
10
5
0
1 2 3 4 5 6 7
Normal
Tumor


A
76N (Normal)
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
MDA-MB-157 (Cancer)
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
cyclin E
cyclin A
B
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
cyclin E-
H1 Kinase
cdk2-
H1 Kinase
C
H1 kinase activity, log10 cpm
1000000
100000
10000
1000
100
0 3 6 9 12 15 18 21 24 27 30 33
Time, hrs
cyc E, kinase
cdk2, kinase
H1 kinase activity, log10 cpm
1000000
100000
10000
1000
100
0 4 8 12 16 20 24 28 32 36 40 44
Time, hrs
D
Cells in G2/M and S, % of Control
100
75
50
25
0
0 3 6 9 12 15 18 21 24 27 30 33
Time, hrs
Cells in G1, G2/M and S, % of Control
100
75
50
25
0
0 4 8 12 16 20 24 28 32 36 40 44
Time, hrs

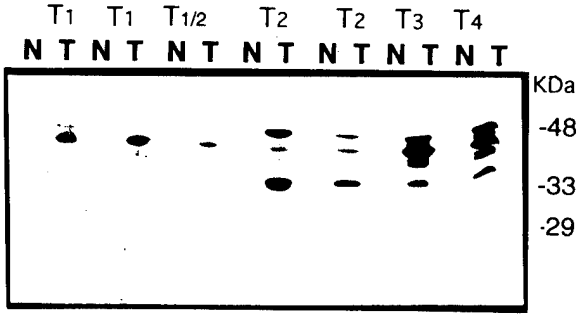

A
M 1 2 M
bp
1344
1096
B
L1
5'
WT CYCLIN E
3'
R1
Δ9 CYCLIN E
66 76
Δ148 CYCLIN E
999
1148
3
C
35 S-Methionine Labelled Cyclin E Proteins
wt Δ9 Δ148
50
33
27
D
Polyclonal Antibody to Cyclin E
wt Δ9 Δ148
50
33
27

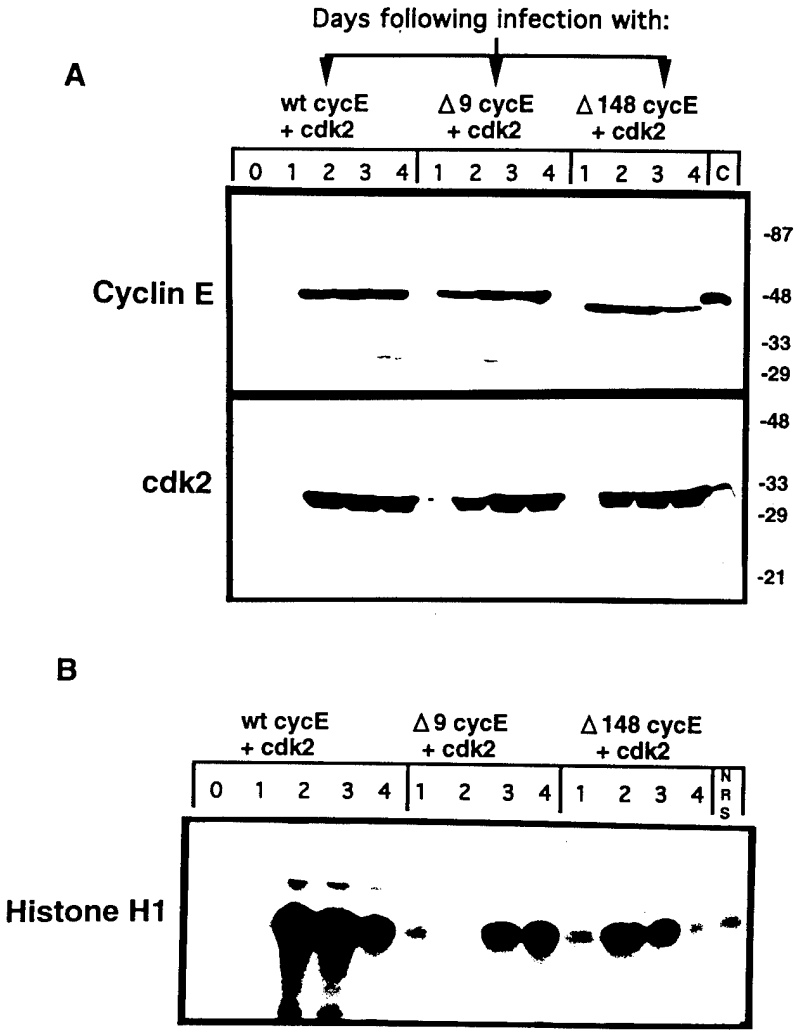

A
Normal
Tumor
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
bp
cyclin E
coding region
-1353
-1078
-872
-603
-310
B
Normal
Tumor
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
bp
cycE Δ9
specific
-1353
-872
1078
-603
-310
C
Normal
Tumor
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
bp
cycE Δ148
specific
1353
1078
-872
-603
-310

A


T1 T1 T1/2 T2 T2 T3 T4
M N T N T N T N T N T N T N T M bp
-1353
-1078
-872
-603
-310

B


T1 T1 T1/2 T2 T2 T3 T4
N T N T N T N T N T N T N T
KDa
-48
-33
-29


Days following infection with:
A
wt cycE + cdk2
Δ9 cycE + cdk2
Δ148 cycE + cdk2
0 1 2 3 4 1 2 3 4 1 2 3 4 C
Cyclin E
cdk2
-87
-48
-33
-29
-48
-33
-29
-21
B
wt cycE + cdk2
Δ9 cycE + cdk2
Δ148 cycE + cdk2
0 1 2 3 4 1 2 3 4 1 2 3 4 N R S
Histone H1

Oncogene (1995) 11, 941-950

Deregulation of cyclin E in breast cancer

Khandan Keyomarsi, Darryl Conte Jr, Wendy Toyofuku and M Pat Fox

Division of Molecular Medicine, Laboratory of Diagnostic Oncology, Wadsworth Center, Albany, New York 12201, USA

Cyclin E, a regulatory subunit of cyclin dependent kinase-2, is thought to be rate limiting for the G1/S transition during the mammalian cell cycle. Previously, we showed severe alterations in cyclin E protein expression in human mammary epithelial cell lines and in surgical material obtained from patients with various malignancies. To understand the functional basis of these alterations we analyse here the regulation of cyclin E in breast cancer cells. We find that while cyclin E protein and its associated kinase activity in normal cells are cell cycle regulated, in tumor cells it remains in an active complex throughout the cell cycle. We also analysed cyclin E for possible deletions which could result in its constitutive function and found two novel truncated variants in its coding region. These variant forms of cyclin E were detected in several normal and tumor cell lines and tissue specimens. However, Western blot analysis indicated that only the multiple isoforms of cyclin E protein were expressed in tumor but not the normal tissue specimen, suggesting post transcriptional regulation of cyclin E. Lastly, *in vitro* analyses indicated that these truncated variant forms of cyclin E are biochemically active in their ability to phosphorylate histone H1. Collectively these observations suggest the presence of more than one form of cyclin E mRNA in all cells, normal and tumor. Once translated in tumor cells, the protein products of these truncated forms could give rise to a constitutively active form of cyclin E containing complexes.

Keywords: breast cancer; cell cycle; cyclin E; alternative splicing

Introduction

Cyclins are prime cell cycle regulators and central to the control of cell proliferation in eukaryotic cells via their association with and activation of cyclin-dependent protein kinases 1-7 (cdks) (reviewed in, Elledge and Spottswood, 1991; Heichman and Roberts, 1994; Hunter and Pines, 1994; King *et al.*, 1994; Nurse, 1994; Sherr, 1994; Morgan, 1995). Cyclins were first identified in marine invertebrates as a result of their dramatic cell cycle expression patterns during meiotic and early mitotic divisions (Evans *et al.*, 1983; Swenson *et al.*, 1986; Standart *et al.*, 1987; Sherr, 1993). Several classes of cyclins have been described and are currently designated as cyclins A-H, some with multiple members (reviewed in Draetta, 1994). Cyclins can be distinguished on the basis of conserved sequence motifs, patterns of appearance and apparent functional roles during specific phases and regulatory points of the cell cycle in a variety of species.

The connection between cyclins and cancer has been substantiated with the D type cyclins (Hunter and Pines, 1991; Sherr, 1993; Draetta, 1994; Hunter and Pines, 1994). Cyclin D1 was identified simultaneously by several laboratories using independent systems: It was identified in mouse macrophages due to its induction by colony stimulating factor 1 during G1 (Matsushime *et al.*, 1991); in complementation studies using yeast strains deficient in G1 cyclins (Lew *et al.*, 1991; Xiong *et al.*, 1991); as the product of the *bcl*-1 oncogene (Withers *et al.*, 1991) and as the PRAD1 proto-oncogene in some parathyroid tumors where its locus is overexpressed as a result of a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene (Matsushime *et al.*, 1991; Motokura *et al.*, 1991, Motokura and Arnold, 1993; Quelle *et al.*, 1993). In centrocytic B cell lymphomas cyclin D1 (PRAD1)/BCL1 is targeted by chromosomal translocations at the BCL1 breakpoint, t(11;14) (q13;q32) (Rosenberg *et al.*, 1991a,b). Furthermore, the cyclin D1 locus undergoes gene amplification in mouse skin carcinogenesis, as well as in breast, esophageal, colorectal and squamous cell carcinomas (Lammie *et al.*, 1991; Jiang *et al.*, 1992, 1993b; Bianchi *et al.*, 1993; Buckley *et al.*, 1993; Leach *et al.*, 1993). Several groups have examined the ability of cyclin D1 to transform cells directly in culture with mixed results (Hinds *et al.*, 1992, 1994; Jiang *et al.*, 1993a; Quelle *et al.*, 1993; Rosenwald *et al.*, 1993; Sherr, 1993; Lovec *et al.*, 1994; Musgrove *et al.*, 1994; Resnitzky *et al.*, 1994). However, the overexpression of cyclin D1 was recently observed in mammary cells of transgenic mice and results in abnormal proliferation of these cells and the development of mammary adenocarcinomas (Wang *et al.*, 1994). This observation strengthens the hypothesis that the inappropriate expression of a G1 type cyclin may lead to loss of growth control.

Recently, we and others have reinforced the linkage between oncogenesis and the cell cycle by correlating the deranged expression of cyclins to the loss of growth control in breast cancer (Buckley *et al.*, 1993; Keyomarsi and Pardee, 1993). Using proliferating normal *vs* human tumor breast cell lines in culture as a model system, we have described several changes that are seen in all or most of these lines. These include increased cyclin mRNA stability, resulting in overexpression of mitotic cyclins and cdc2 RNAs and proteins in 9/10 tumor lines, leading to the deranged order of appearance of mitotic cyclins prior to G1 cyclins in synchronized tumor cells.

The most striking abnormality in cyclin expression we found, was that of cyclin E. Cyclin E protein not only was overexpressed in 10/10 breast tumor cell lines but it was also present in lower molecular weight

Correspondence: Khandan Keyomarsi
Received 31 March 1995; revised 8 June 1995; accepted 8 June 1995

isoforms than that found in normal cells (Keyomarsi and Pardee, 1993). We directly examined the relevance of cyclin derangement to *in vivo* conditions, by measuring the expression of cyclin E protein in tumor samples *vs* normal adjacent tissue obtained from patients with various malignancies (Keyomarsi *et al.*, 1994). These analyses revealed that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alteration in cyclin E protein expression independent of the S-phase fraction of the samples. In addition, the alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as PCNA or c-*erb*B2. These observations strongly suggested the use of cyclin E as a new prognostic marker.

In this report, we have further characterized the alterations of cyclin E in breast cancer. We show that while cyclin E is cell cycle regulated in normal cells it is present constitutively and in an active cdk2 complex in synchronized populations of breast cancer cells. We also identify two novel truncated variant forms of cyclin E mRNA as detected by RT-PCR which are ubiquitously detected in normal and tumor cells and tissues. These variant forms of cyclin E can give rise to an active cyclin/cdk2 complex *in vitro*, but they do not seem to be translated in normal cells.

Results

Elevated cyclin E associated kinase activity in breast cancer cells

To test the hypothesis that the altered expression pattern of cyclin E protein found in tumor cell lines and tissue samples (Keyomarsi *et al.*, 1994) is associated with increased cyclin E kinase activity, we compared cyclin E expression and activity in two normal *vs* five breast cancer cell lines (Figure 1). The two normal cell lines are the normal cell strain, 76N (Figure 1, lane 1), obtained from reduction mammoplasty and a near diploid immortalized cell line MCF-10A (Figure 1, lane 2) (Soule *et al.*, 1990). 76N is a mortal cell strain since it rapidly proliferates (doubling time of 24–27 h) for multiple passages before senescence at around passage 20 (Band and Sager, 1989). The MCF-10A cell line is a spontaneously immortalized human breast epithelial cell line which can be cultured indefinitely. This cell line has no tumorigenicity potential but retains characteristics of a normal breast epithelial cell line (Soule *et al.*, 1990).

We examined the pattern of cyclin E protein expression in normal *vs* tumor cell lines using monoclonal and polyclonal antibodies to cyclin E on Western blots (Figure 1A). Similar immunoblot banding patterns were obtained with either the monoclonal or polyclonal antibody to cyclin E, confirming the specificity of the multiple bands. However, the patterns of cyclin E protein expression was different between normal and tumor cells. Both cyclin E antibodies recognized one major protein migrating at ~50 kDa and two much less abundant lower molecular weight forms, in the two normal cell lysates. In the tumor cell lysates on the other hand, the

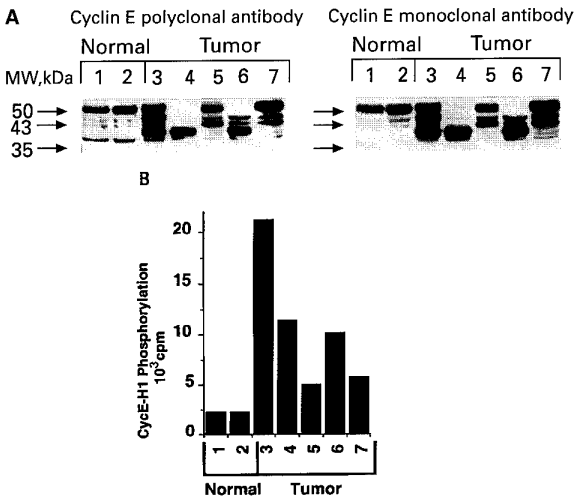


Figure 1 Correlation of cyclin E protein(s) to cyclin E associated kinase activity (**A**) Western blot analysis of cyclin E expression in normal *vs* tumor cells using two different cyclin E antibodies. Whole cell lysates were extracted from the seven cell lines, (100 µg of protein extract/lane), run on a 10% acrylamide gel and blotted as described in Materials and methods. Lane 1, 76N normal human mortal breast epithelial cell strain; lane 2, MCF-10A normal immortalized human breast epithelial cell line; (lanes 3–7 are all human breast cancer cell lines) lane 3, MDA-MB-157; lane 4, MDA-MB-436; lane 5, ZR75T; lane 6, SKBR3; lane 7, MCF-7. The 50 kDa arrowhead points to the cyclin E protein of the predicted size. The other arrowheads point to the additional cyclin E isoforms observed in the tumor cell lines ranging in molecular weight from 35 to 43 kDa. Molecular mass standards were used in each gel to estimate the position of each band. (**B**) Cyclin-E associated histone H1 kinase activity. Equal amounts of proteins from cell lysates were immunoprecipitated with anti-cyclin E coupled to protein A beads using histone H1 as substrate. The associated kinase activities were quantified by scintillation counting

same antibodies recognized three (lane 3), two (lanes 5–7) or one (lane 4) additional and highly abundant isoforms of cyclin E protein that in each case revealed a different pattern from that of the normal cells.

We next analysed the cyclin E associated protein kinase activity in all cells by measuring the phosphorylation of histone H1 in immunoprecipitates made with the polyclonal antibody to cyclin E (Figure 1B). In all of the tumor cell lysate immunoprecipitates, the activity levels of cyclin E-associated kinase were significantly higher than that of both normal cells. For example, in MDA-MB-436 and SKBR3 tumor cell lines (lanes 4 and 6) which express only the lower molecular weight isoforms of cyclin E protein, the associated kinase activity was sixfold greater than that of the normal cells which express mainly the high molecular weight, 50 kDa, form of cyclin E protein. Similarly, the other tumor lines containing altered patterns of cyclin E expression, had significantly higher cyclin E-associated H1-kinase activity as compared to the normal cell strains.

Lack of cell cycle regulation of cyclin E in breast cancer cells

In one tumor line, MDA-MB-157 (Figure 1, lane 3), the level as well as the associated kinase activity of cyclin E protein was the highest of all the tumor cell lines examined. Previous studies (Keyomarsi and

Pardee, 1993) showed that this overexpression is in part due to an eightfold amplification of the cyclin E gene and 64-fold overexpression of its mRNA in this cell line. The cyclin E gene is amplified in tandem and is not associated with gross genomic rearrangements (data not shown). To investigate whether the signals required for normal regulation of cyclin E expression are altered or lost in tumor cells, the cell cycle expression of cyclin E protein and its associated kinase activities in the MDA-MB-157 cell line were compared to normal mammary epithelial 76N cells (Figure 2).

Both cell lines were synchronized in the G1/S border by double thymidine block. Synchrony of both cell types at several times after release from the block was monitored by flow cytometry * (Figure 2D). At various times after release from treatment for synchronization, cells were harvested and extracted proteins were analysed on Western blots with antibodies to cyclins E and A (Figure 2A). In normal 76N cells, the pattern of expression of cyclin E and cyclin A proteins is consistent with that seen for other normal cell types with levels rising prior to S phase and oscillating thereafter in the cell cycle (Koff *et al.*, 1992). In addition there is only one major form (i.e., 50 kDa) of cyclin E protein detected and there is a shift in the timing of when cyclin E *vs* cyclin A appears in the cell cycle of these normal epithelial cells. However, in the tumor cells, cyclin E protein does not appear to be cell cycle regulated and multiple isoforms of the protein are also present with similar signal intensities and banding patterns during the time intervals examined. In addition when these tumor cells are synchronized by other agents, such as Lovastatin (Keyomarsi *et al.*, 1991), cyclin E expression is also constitutive throughout the cell cycle, resembling a pattern identical to that shown in Figure 2A (data not shown). In the same tumor cell extracts, cyclin A protein is cell cycle regulated with peak levels coinciding with peak S and early G2/M phase. Hence, it appears that in this tumor cell line, cyclin E is abnormally regulated during the cell cycle.

In order to compare the kinase activity associated with cyclin E and cdk2 in normal and tumor cells, we measured the phosphorylation of histone H1 in immunoprecipitates prepared from synchronous cell extracts using antibody to either cyclin E or cdk2 (Figure 2B). There were two significant differences found between normal and tumor cells: First, in the length of time which an active cyclin E/cdk2 complex is present and secondly in the amount of kinase activity associated with cyclin E *vs* cdk2 during the normal and tumor cell cycles. In normal cells, both cyclin E associated kinase and cdk2 activities are cell cycle regulated, coinciding with the levels of cyclins E and A protein expression (Figure 2A). In addition, the cdk2 activity is one order of magnitude (i.e. 10-fold) higher than cyclin E associated activity, consistent with cdk2's ability to form an active complex with other cyclins besides cyclin E in normal cells (Figure 2B). Hence, cyclin E in these normal cells is indeed cell cycle regulated and the signals required for such regulation are intact both at the protein expression level and kinase activity.

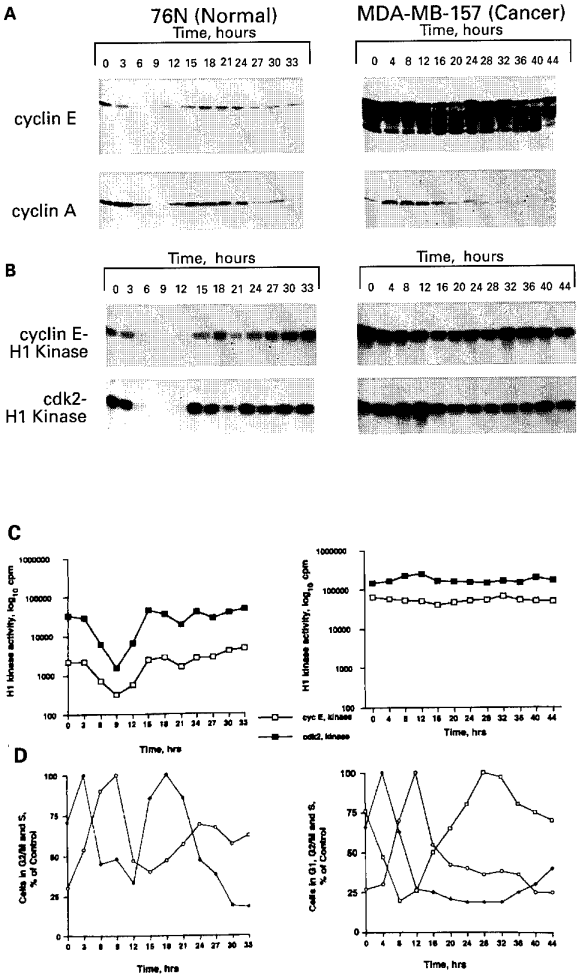


Figure 2 Expression of cyclin E in synchronized normal 76N and tumor MDA-MB-157 breast cells. Both cell types were synchronized by double thymidine block procedure (see Materials and methods). At the indicated times following release from double thymidine block, cell lysates were prepared and subjected to (**A**) Western blot and (**B**) Histone H1 kinase analysis. Protein (50 μg) for each time point was applied to each lane of a 10% acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity purified polyclonal antibodies. The blots were stripped between the two assays in 100 mM β-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8) and 2% SDS for 30 min at 55°C. For Histone H1 kinase activity, equal amount of proteins (600 μg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cyclin E (polyclonal) or anti-CDK2 (polyclonal) coupled to protein A beads using histone H1 as substrate. Panel B is the autoradiogram of the histone H1 SDS–PAGE gel and (**C**) shows the quantification of the histone H1 associated kinase activities by scintillation counting. Open symbols correspond to cyclin E associated kinase activity and closed symbols correspond to cdk2 activity (**D**). At various times after release from double thymidine block, aliquots were removed and subjected to flow cytometry analysis. Cells in S phase (◆), G2/M phase (○) and G1 phase (□), are expressed as percent of control, where control is equal to the time when the maximum number of cells enter each phase of the cell cycle

*The doubling times of the normal 76N and tumor MDA-MB-157 cells are 27 and 36 h, respectively, and their DNA content distribution in different cell cycle phases are as follows: 76N- G1 (75%), S (4%) and G2/M (21%); MDA-MB-157-G1 (56%), S (13%) and G2/M (31%).

In tumor cells, on the other hand, cyclin E is not cell cycle regulated and remains in a catalytically active complex throughout the cell cycle resulting in a constitutive pattern of histone H1 phosphorylation. The basal levels of cyclin E associated kinase activity during the tumor cell cycle, at any time interval examined, are at least 20 times higher than that of the normal cells (Figure 2C). Cdk2, a kinase which binds to both cyclin E and A, is also constitutively active during the cell cycle. However, cdk2 activity in this tumor cell line is only twofold higher than cyclin E associated kinase activity, presumably due to the abundance of cyclin E protein which is capable of sequestering cdk2. When cyclin A protein levels are induced in the tumor cells, there is only a 30% additional induction in cdk2 associated activity. These observations suggest that cyclin E protein, which is constitutively expressed in the cell cycle of tumor cells, also results in an active kinase complex throughout the cell cycle. Furthermore, since the same cyclin-dependent kinase can be regulated by both cyclins E and A, increased levels of cyclin E may overcompensate for cyclin A regulation, again resulting in a constitutively active and abundant cyclin E/cdk2 complex.

Isolation of variant forms of cyclin E transcripts

In an attempt to determine the presence of any potential alterations in the cyclin E gene in MDA-MB-157, we amplified the entire cyclin E coding region of this cell line by reverse transcription-polymerase chain reaction amplification (RT-PCR), cloned these products and analysed their DNA sequence (Figure 3). Using a pair of primers flanking the coding sequence of cyclin E gene, we observed at least two distinct PCR products ranging in size from 1.0 to 1.2 kb from the MDA-MB-157 RT template (Figure 3A). The product from the control (cyclin E plasmid DNA) was of 1.2 kb, corresponding to the full length cyclin E cDNA isolated from a HeLa cDNA library (Koff *et al.*, 1991; Lew *et al.*, 1991). We cloned the RT-PCR products from the MDA-MB-157 cell line and confirmed their identity by Southern blotting and by DNA sequencing (data not shown). Three independent RT-PCR reactions were performed on freshly isolated RNA from this cell line. Fifteen clones from each RT-PCR reaction were examined further. Sequence analyses revealed two types of truncated variants of the cyclin E gene, as well as an unequivocally normal sequence, from the MDA-MB-157 cell line (Figure 3).

The PCR products containing these two truncated variants were termed cyclin E-Δ9 and cyclin E-Δ148 (Figure 3B). The alteration in clone cyclin E-Δ9 is a 9 base pair in-frame deletion of nucleotides 67–75 at the 5′ end of the gene, while the alteration in clone cyclin E-Δ148 is a 148 base pair deletion of nucleotides 1000–1147 at the 3′ end of the gene resulting in a frame shift transcript. Curiously, the 148 bp deletion in cyclin E-Δ148 clone disrupts the PEST sequence motif of the gene, which is thought to be important for its role in degradation of the protein product (Koff *et al.*, 1991; Lew *et al.*, 1991). The relative positions of these two newly identified truncations to the wild type sequence of cyclin E are shown in Figure 3B. We performed *in vitro* translation studies on these clones

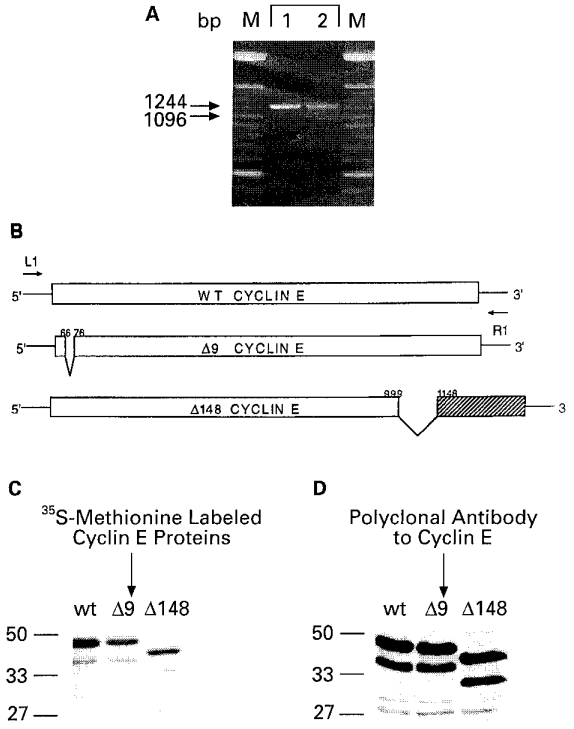


Figure 3 Identification and *in vitro* translation of cyclin E truncated transcripts. (**A**) PCR amplified cyclin E coding sequence using primers (L1CYCE and R1CYCE) flanking the entire coding region of cyclin E. Lane 1: Molecular weight standards; lane 2: control template DNA, a plasmid containing a wild type cyclin E coding sequence; lane 3: RT-PCR amplification of cyclin E using RNA from MDA-MB-157; lane 4: Molecular weight standards. PCR conditions were carried out as described in Materials and methods. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Molecular weight markers in base pairs are indicated (left). (**B**) Relative position of cyclin E Δ9 and Δ148 deletions to the wild type cyclin E sequence. The two arrows flanking the cyclin E coding region refer to the position of R1 (i.e., R1CYCE) and L1 (i.e.,L1CYCE) oligonucleotides used for the RT-PCR reactions. (**C**) The cDNAs of cyclin E clones were subcloned into PCR II vector and transcribed and translated *in vitro* using T7 RNA polymerase-rabbit reticulocyte lysate system in the presence of [^{35}S]methionine and products were analysed on a 10% SDS–PAGE followed by autoradiography (**D**) The *in vitro* translated cyclin E protein products from the three different clones (in the absence of radioactivity) were subjected to Western blot analysis and hybridized to a polyclonal antibody to cyclin E

using T7 RNA polymerase (Figure 3C and D). RNA was translated in the presence of [^{35}S]methionine using a rabbit reticulocyte lysate, analysed by SDS–PAGE and visualized by autoradiography. Cyclin E-wt and cyclin E-Δ9 protein products showed very similar electrophoretic mobilities (Figure 3C). [On a sequencing length SDS–PAGE, gel however, we were able to detect the slight (3 amino acids) molecular weight difference between the two clones (data not shown)]. Cyclin E-Δ148 gives rise to a protein product which is ~5 kD smaller than the cyclin E-wt, which would correspond to the loss of the 50 amino acids. To confirm that the protein products from *in vitro* translation reactions were indeed cyclin E, the cDNAs of the three different clones were transcribed and translated in the presence of unlabeled methionine and the products were subjected to Western blot analysis

(Figure 3D). The protein products from cyclin-wt, Δ9 and Δ148 clones reacted strongly with the polyclonal antibody to cyclin E, suggesting that the *in vitro* translated products of these clones are truncated forms of cyclin E. Interestingly, all the clones gave rise to two major protein products, migrating at ~45 and ~38 kD for cyclin E-wt and Δ9 clones and ~40 and ~33 kD for cyclin E-Δ148 clone. It is not clear at this point whether the lower molecular weight protein product is a result of proteolytic cleavage, or result of translation initiation from a methionine site further downstream in the coding region.

Expression of cyclin E truncated variants in normal vs *tumor cells and tissue samples*

Since these two truncated forms of cyclin E cDNA were isolated from one tumor-derived cell line, we investigated the generality of expression of the cyclin E variants in a panel of 13 breast epithelial cell lines (Figure 4A). These cell lines included three normal mortal cell strains (lanes 1–3), one normal immortalized cell line (lane 4) and nine tumor-derived breast cell lines (lanes 5–13). These analyses revealed the presence of multiple transcripts of cyclin E in all cell lines examined. However, no distinct differences were observed in their pattern of expression between normal *vs* tumor cell lines. Furthermore, sequence analysis of a cloned RT-PCR product of 76N normal cells revealed that the major transcript found in this normal cell strain is the Δ148 variant of cyclin E previously identified in MDA-MB-157 tumor cell line (data not shown). These observations indicate that the Δ148 RNA is expressed in all cells examined at an apparently higher level than the wild type species of cyclin E RNA. To examine the specific expression of Δ9 and Δ148 in each cell line, we performed RT-PCR using primers that spanned the deleted sequences, such that only those cell lines containing cyclin E transcripts harboring these deletions would give rise to products. These analyses show that the Δ9 variant form of cyclin E is abundantly present in three cell lines, two of which are normal cell strains and one is the MDA-MB-157, the original cell line this variant form was isolated from (Figure 4B). In addition we find that the Δ148 is present in all cell lines examined (Figure 4C), confirming our previous observation that this variant form of cyclin E is the major transcript found in these cells (Figure 4A).

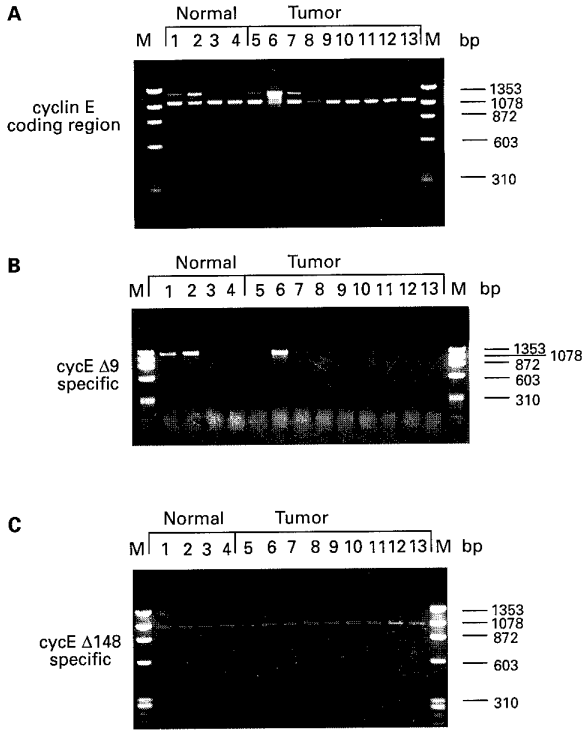


Figure 4 RT-PCR amplification of cyclin E Δ9 and Δ148 in normal and tumor-derived breast epithelial cell lines. RT-PCR amplification of cyclin E coding sequence from normal and tumor-derived breast epithelial cell lines using (**A**) primers (L1CYCE and R1CYCE) flanking the coding region of cyclin E and amplifying wild type cyclin E sequences, as well as those containing internal deletions, (**B**) primers (LMEMARK3 and R1CYCE) spanning the Δ9 deletion and amplifying only those cyclin E sequences harboring the Δ9 internal deletion of cyclin E and (**C**) primers (L1CYCE and RMEMARK3) spanning the Δ148 internal deletion and amplifying only those cyclin E sequences containing the Δ148 deletion. The cell lines used are as follows: Lane 1, 70N; lane 2, 81N; lane 3, 76N; lane 4, MCF-10A; lane 5, MCF-7; lane 6, MDA-MB-157; lane 7, MDA-MB-231; lane 8, MDA-MB-436; lane 9, T47D; lane 10, BT-20T; lane 11, HBL-100; lane 12, Hs578T and lane 13, ZR75T. Normal cells are represented in lanes 1–4 and tumor-derived cell lines in lanes 5–13; M, Molecular weight size markers

In order to apply our findings from culture studies to the *in vivo* condition, we examined whether the truncated cyclin E transcripts were also expressed in tumor tissue specimens. We performed RT-PCR using RNA isolated from seven paired samples of human breast carcinoma and normal adjacent tissue (NAT) which are presented according to increased clinical stage (Figure 5). For this experiment, we used primers flanking the entire coding region of cyclin E in order to detect all variants of cyclin E which could contain deletions in the coding region. The RT-PCR products from NAT and tumor tissue samples ranged in size from 1.0 to 1.2 kb (Figure 5A), which are consistent with products obtained with cultured breast cells (Figure 4). Surprisingly, we found that not only did both NAT and tumor tissue samples express similar RT-PCR products corresponding to the cyclin E variants, but that no distinct difference could be found among paired samples as the clinical stage of the disease increases. On the other hand, when we subjected whole cell lysates prepared from these tissue specimens to Western blot analysis, we did observe cyclin E protein alterations which increased qualitatively and quantitatively as the stage of the disease increased. In high staged tumor samples, an antibody to cyclin E reacted strongly with at least three overexpressed proteins ranging in size from 35 to 50 kDa, while in the NAT samples, one major protein of 50 kDa was present at very low levels, consistent with our previous observations (Keyomarsi *et al.*, 1994). Collectively these observations suggest that at the level of RNA there are no apparent differences between normal and tumor cells or between tissue samples in their ability to express the alternate transcripts of cyclin E. However, the alteration in cyclin E protein observed exclusively in tumor cells,

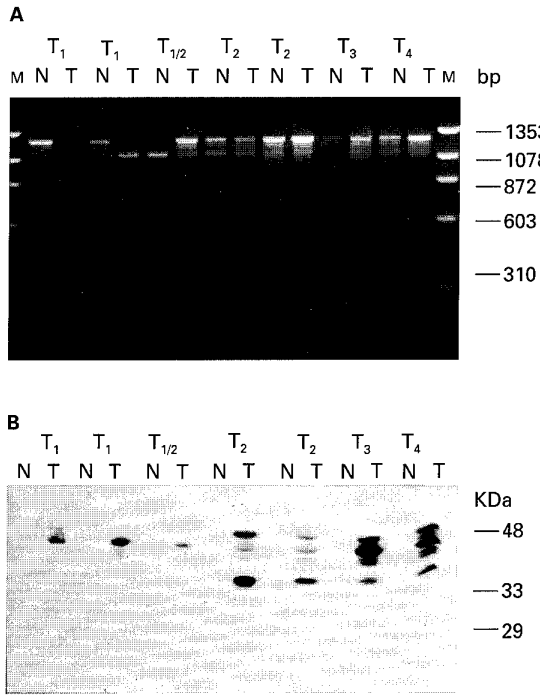


Figure 5 Comparison of RT-PCR amplified products of cyclin E with their expression in breast cancer specimens. (**A**) RT-PCR amplification of cyclin E coding sequence using total RNA isolated from seven pairs of normal adjacent (NAT) and tumor tissue samples with primers (L1CYCE and R1CYCE) flanking the coding region of cyclin E as described in Figure 4A. (**B**) Western blot analysis of whole cell lysates (100 μg) were prepared from NAT and tumor tissue specimens and probed with a monoclonal antibody to cyclin E. Breast cancer types and histological/tumor grades are as follows: Lanes 1–2, intraductal carcinoma of the breast, Stage T_1, NO, MO, Grade I; Lanes 3–4, invasive well differentiated ductal carcinoma, Stage T_1, NO, MO, Grade I; Lanes 5–6, intraductal carcinoma, Stage $T_{1/2}$, NO, MO, Grade I; Lanes 7–8, invasive and intraductal carcinoma, Stage T_2, NO, MO, Grade II; Lanes 9–10, *in situ* and infiltrating ductal carcinoma, Stage T_2, N1, MO, Grade II/III; Lanes 11–12, infiltrating ductal carcinoma, Stage T_3, NO, MO, Grade II/III; Lanes 13–14, invasive ductal carcinoma, Stage T_4, NO, MO, Grade III. Molecular mass standards were used on each gel to estimate the position of each band

likely occurs post transcriptionally or translationally to result in various forms of the protein detected in tumor but not normal cells or tissues.

Cyclin E truncated variants form biochemically active complexes with cdk2

Based on the evidence that multiple cyclin E transcripts (Figures 4 and 5) are found in normal and tumor cells as well as in tissue samples and that there is an active cyclin E/cdk2 protein complex present throughout the cell cycle of the MDA-MB-157 cell line (Figure 2), we asked whether these alternate transcripts of cyclin E can give rise to a biochemically active product. To investigate this question, we overexpressed cyclin E and cdk2 in insect cells using the baculovirus expression system (Figure 6). Insect cells were co-infected with the recombinant baculovirus containing cdk2 and either cyclin E- wild type (cycE-wt), cyclin E-Δ9, (cycE-Δ9), or cyclin E-Δ148 (cycE-Δ148) cDNAs (Figure 6). At the indicated times (i.e. days) following infection, cell extracts were collected, homogenized and subjected to Western blot and histone H1 kinase analysis. Western blot analysis shows that there were similar levels of expression of the three cyclin E variants and cdk2 in the infected sf9 cells within one day of infection and thereafter during the course of experiment (Figure 6A). H1 kinase analysis reveal that when the cyclin E-wt/cdk2 co-infected insect cell lysates were immunoprecipitated with an antibody to cdk2, the immunoprecipitates were capable of phosphorylating histone H1 within one day of infection and an active cyclin E/cdk2 complex persisted throughout the experiment (Figure 6B, lanes 1–4). In insect cells co-infected with the two truncated variants of cyclin E, similar results were obtained illustrating that the complex which cycEΔ9 (Figure 6C, lanes 5–8) or cycEΔ148 (Figure 6C, lanes 9–12) formed with cdk2 is also active and is capable of phosphorylating histone H1. However a lower degree of activation was found compared to that with the cyclin E wild type complex. There was a twofold difference in the ability of the cycEΔ9/cdk2 or cycEΔ148/cdk2 to phosphorylate histone H1 when

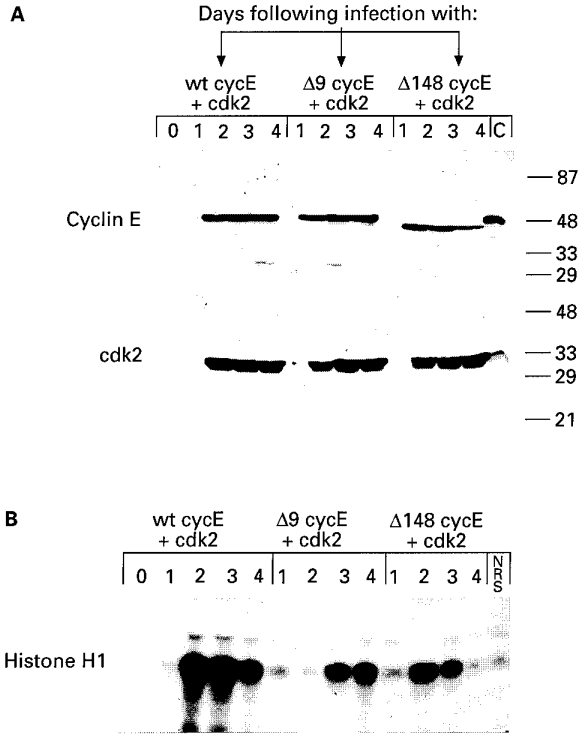


Figure 6 Activation of cdk2 by cyclin E wild type and its truncated variants in insect cells. Cell lysates were prepared from insect cells co-infected with baculovirus containing the different cyclin E constructs and cdk2 at the indicated time intervals (days) following co-infection. (**A**) Equal amounts (50 μg) of protein were added to each lane; the gel was then subjected to Western blot analysis with polyclonal antibody to cyclin E or to cdk2. C: Control lane corresponding to 50 μg of extracts from insect cells infected with either cyclin E wt alone, or cdk2 alone baculovirus (**B**) Histone H1 kinase assays were also performed on the same cell extracts by immunoprecipitating equal amount of cell lysate with polyclonal antibody to cdk2 coupled to protein A beads using histone H1 as substrate. The autoradiogram of the histone H1 SDS–PAGE gel is depicted. NRS: A control immunoprecipitate performed with *N*ormal *R*abbit *S*erum in place of cdk2 antibody

compared to cyclin E wt/cdk2. These *in vitro* analyses suggest that once the cyclin E variant transcripts are translated, the protein products can give rise to a functionally active cyclin E complex capable of phosphorylating substrates such as histone H1.

Discussion

In an attempt to understand the relationship between the cell cycle and cancer, many laboratories have investigated the role cyclin/cdk complexes play in cancer. While cyclins D and A have been implicated in tumorigenesis, the role of other cyclins have been elusive and limited mainly to observations. Cyclin E is an interesting case since it shows an altered pattern of expression in all breast cancer cell lines and tumor tissue samples we have examined to date (Keyomarsi *et al.*, 1994). The cyclin E alterations include over-expression of the authentic-sized protein as well as expression of lower molecular weight isoforms found in tumor cells or tissues. We set out to decipher the mechanism responsible for these alterations by initially correlating the activity of cyclin E/cdk2 complexes with the expression pattern and level of cyclin E protein, both in exponentially growing and synchronized population of normal *vs* tumor cells. We find that regardless of which combination of the cyclin E (50 kDa) and its lower molecular weight isoforms are expressed in these tumor cell lines, the associated kinase activity is much higher in tumor than normal cells. Furthermore, we find that in synchronized populations of tumor cells, cyclin E is present in altered forms throughout the cell cycle and the kinase activity associated with it, or with cdk2, is also constitutively active. In addition the abundant and constitutive expression of cyclin E in these tumor cells result in sequestering of cdk2 away from other cyclins, such as cyclin A. This suggests that, there is a cyclin E/cdk2 complex which is abundantly and uniformly active in the tumor but not the normal cell cycle.

In order to determine whether the multiple forms of the cyclin E protein detected in tumor cells originate from different transcripts of cyclin E RNA, we performed RT-PCR and found two different truncated variants of cyclin E (i.e. Δ9 and Δ148) expressed in MDA-MB-157 tumor cell line. Further analysis revealed that the most intriguing feature of the Δ9 and Δ148 variant forms of cyclin E is that there is no distinct difference in their mRNA expression in normal *vs* tumor cells or tissue samples. In addition there is little correlation between expression of these cyclin E variants at the level of RNA *vs* protein. Yet, we show here that at the level of protein in tumor cells, (a) cyclin E isoforms ranging in size from 35 to 50 kDa are abundantly expressed (Figures 1, 2 and 5) and that (b) these protein isoforms of cyclin E are not subject to cell cycle regulation and may constitutively interact with cdk2 resulting in an active complex (Figure 2). Lastly (c) we also show that once Δ9 and Δ148 transcripts of cyclin E are allowed to express their protein products, the resulting proteins can bind to cdk2 and form active complexes *in vitro* (Figure 6). Collectively, based on these observations we suggest that the multiple protein isoforms of cyclin E detected in tumor cells are a result of altered post-transcriptional and/or translational regulation of cyclin E mRNAs. Hence, there may be a translational fidelity that has been altered/lost in tumor cells, allowing for the translation of these truncated variants of cyclin E to occur and once translated, they can form active complexes with cdk2 throughout the cell cycle (see Figures 2 and 6). Alternatively, there may be post-translational modification of cyclin E which is also altered or lost in tumor cells. The presence of lower molecular weight protein isoforms of cyclin E, barely detectable in normal cell lysates (Figure 1, lanes 1 and 2) could also suggest that these isoforms of cyclin E are in fact translated in normal cells as well, but they are rapidly degraded. In tumor cells, the protein turnover is much longer and as a result we can readily detect lower molecular weight isoforms of cyclin E which are highly abundant and functionally active.

One possibility for the presence of multiple transcripts of cyclin E is due to alternative splicing. Precedent for alternative splicing of cyclin E has recently been reported by Ohtsubo *et al.* (1995) where they identified a longer form of cyclin E (cyclin E-L) which contains 15 amino acids at the amino terminus which through alternative splicing, is absent in the original form of cyclin E (cyclin E wt) (Ohtsubo *et al.*, 1995). In addition Sewing *et al.* (1994) also identified another splice variant of cyclin E, termed cyclin Es. Like cyclin E-L and Es, there is a strong possibility that both cyclin EΔ9 and cyclin EΔ148 reported here, are results of alternative splicing as we find potential splice donor and acceptor sites at the deleted junctions of each transcript. However, the cyclin Es variant differs from those we report here in that cyclin Es lacks 49 amino acids within the cyclin box and is 90% less abundant than the wild type cyclin E sequence. This form is unable to associate with cdk2, is inactive in histone H1 kinase assays and is unable to rescue a triple CLN mutation of *S. cerevisiae* (Sewing *et al.*, 1994). Unlike cyclin Es, neither the cyclin EΔ9 nor the Δ148 transcripts disrupts the cyclin box, the consensus region which confers activity by its association to a cdk (Lees *et al.*, 1992). As a result, both Δ9 and Δ148 variants of cyclin E retain the ability to functionally bind to cdk2 and phosphorylate histone H1 in insect cells (Figure 6). The ability of these novel variants of cyclin E to form an active complex with cdk2 has implications for their biological functions. The Δ148 variant has another interesting feature in that the PEST sequence important for its degradation has been disrupted by this 148 base pair deletion. It is possible that the deletion of a PEST sequence may have an effect on turnover of Δ148 cyclin E, allowing it to remain active for a longer duration than the wild type form.

The data presented here suggest that the mechanisms responsible for the presence of the multi isoforms of cyclin E protein in tumor cells may be due to a number of factors, one of which is the altered post-transcriptional or translation regulation of the truncated variants of cyclin E. However a question can be raised whether these two novel variant forms of cyclin E attribute to the cancer phenotype. We present data that these two variants are not a result of deletional mutations in the cyclin E gene as they are expressed in both normal and tumor cells as well as tissue samples. However, they are not readily detected in normal cells

either due to their lack of translation or rapid degradation. There is evidence that when the wild type cyclin E is overexpressed in normal cells the length of G1 is decreased, but cells are not transformed (Ohtsubo and Roberts, 1993; Resnitzky *et al.*, 1994). With the discovery of the cyclin E variants that may be translated in tumor but not normal cells, the oncogenecity of these cyclin E forms can now be directly deciphered. A second question that our data has raised, is whether the lower molecular weight isoforms of cyclin E detected mainly in tumor cells are the protein products of the cyclin EΔ9 and/or Δ148 variant transcripts of cyclin E. By identification of these two variants, we can now utilize them as molecular probes to identify their protein products in tumor cells and tissues. Identification of the multiple protein isoforms of cyclin E will give us insight as to the regulation of this protein, which when complexed with cdk2 is thought to be rate limiting for the G1/S transition during the mammalian cell cycle. With an active cyclin E/cdk2 complex, substrates may be phosphorylated at altered points in the cell cycle resulting in loss of checkpoint control during the progression of G1 to S in tumor cells.

Materials and methods

Cells lines, culture conditions and tissue samples

The culture conditions for 70N, 81N and 76N normal cell strains and MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-436, T47D, BT-20, HBL100, Hs578T, SKBR3 and ZR75T tumor cell lines were described previously (Keyomarsi and Pardee, 1993). MCF-10A is a normal human mammary epithelial cell line which is spontaneously immortalized and does not grow in soft agar and is not tumorigenic in nude mice (Soule *et al.*, 1990). This cell line was obtained from ATCC and is cultured in DFCI-1 (Band and Sager, 1989). All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO_2 and maintained free of Mycoplasma as determined by the MycoTect Kit (Gibco). Snap frozen surgical specimens from patients diagnosed with breast cancer were obtained from the National Disease Research Interchange/Co-operative Human Tissue Network, Eastern Division. The clinical stage and grade of the tissue samples used were obtained from pathology/surgical reports and indicated in the figure legend.

Synchronization and flow cytometry

76N normal mammary epithelial cell strain and MDA-MB-157 tumor cell line were synchronized at the G1/S boundary by a modification of the double thymidine block procedure (Rao and Johnson, 1970). Briefly, 48 h after the initial plating of cells, the medium was replaced with fresh medium containing 2 mM thymidine for either 24 h (76N cells) or for 36 h (MDA-MB-157 cells). This medium was then removed, the cells were washed three times and subsequently incubated in fresh medium lacking thymidine for 12 h (76N cells) or 24 h (MDA-MB-157 cells). Next cells were re-incubated in medium containing 2 mM thymidine, as above, washed with fresh medium and incubated in thymidine free medium for the rest of the experiment. Cells were harvested at the indicated times, cell density was measured electronically using a Coulter Counter (Hialeah, Florida) and flow cytometry analysis was performed. For flow cytometry studies, 10^6 cells were centrifuged at $1000 \times g$ for 5 min, fixed with ice-cold 70% ethanol (30 min at 4°C) and washed with phosphate buffered saline (Crissman and Tobey 1974). Cells were suspended in 5 ml of phosphate-buffered saline containing 10 μg ml^{-1} RNase, incubated at 37°C for 30 min, washed once with phosphate buffered saline and resuspended in 1 ml of 69 μM propidium iodide in 38 mM sodium citrate. Cells were then incubated at room temperature in the dark for 30 min and filtration through a 75 mm Nitex mesh. DNA content was measured on a FACScan flow cytometer system (Becton Dickinson, San Jose, CA) and data were analysed using CELLFIT software system (Becton Dickinson).

Western blot and H1 kinase analysis

Cell lysates and tissue homogenates were prepared and subjected to Western blot anaylsis as previously described (Keyomarsi and Pardee, 1993; Keyomarsi *et al.*, 1994). Briefly, 100 μg of protein from each tissue sample or cell line (for sf9 extracts, 50 μg) were electrophoresed in each lane of a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE) (cyclin E and cyclin A), or a 13% SDS–PAGE (cdk2 and all Sf9 cell extracts) and transferred to Immobilon P. Blots were blocked with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% dried milk, 0.2% Tween overnight at 4°C and were incubated with various primary antibodies diluted in blocking buffer for 3 h. Primary antibodies used were rabbit anti-human cyclin E serum at a dilution of 1:2500 (gift from A Koff and J Roberts, Fred Hutchinson Cancer Research Center), monoclonal antibody HE12 to cyclin E at a dilution of 1:10 (a gift of E Lees and E Harlow, Massachusetts General Hospital [MGH] Cancer Center), affinity-purified rabbit anti-human $p33^{cdk2}$ kinase antibody at a dilution of 1:2000 (a gift from L-H Tsai and E Harlow, MGH Cancer Center) and affinity-purified rabbit anti-human cyclin A antibody at a dilution of 1:20 000 (a gift from JW Harper, Baylor College of Medicine). Following primary antibody incubation, the blots were washed and incubated with either goat anti-mouse or anti-rabbit horseradish peroxidase conjugate at a dilution of 1:5000 in blocking buffer for 1 h and finally washed and developed with detection reagents (ECL) supplied by Amersham biochemicals. ECL exposures for all Western blots are of similar duration, i.e. 1–10.

For H1 kinase assays, 250 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with either polyclonal antibody to cyclin E or CDK2 in lysis buffer containing 50 mM Tris HCl pH 7.5, 250 mM NaCl, 0.1% NP-40, 25 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 1 mM benzamidine, 10 μg ml^{-1} soybean trypsin inhibitor, 0.5 mM PMSF, 50 mM NaF, 0.5 mM Sodium Ortho-Vanadate. The protein/antibody mixture was incubated with protein A Sepharose for 1 h and the immunoprecipitates were then washed twice with lysis buffer and four times with kinase buffer (50 mM Tris HCl pH 7.5, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg ml^{-1} BSA). Immunoprecipitates were then incubated with kinase buffer containing 5 μg histone H1, 60 μM cold ATP and 5 μCi of [^{32}P]γATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then analysed on a 13% SDS–PAGE gel. The gel was then stained, destained, dried and exposed to X-ray film. For quantitation, the protein bands corresponding to histone H1 were excised and radioactivity was measured by scintillation counting.

Reverse transcription-polymerase chain reaction amplification (RT-PCR)

RNA was isolated from cell lines and tissue samples as previously described (Keyomarsi and Pardee, 1993). To

remove chromosomal DNA contamination from RNA, 50 μg of total cellular RNA was incubated for 30 min at 37°C with 10 units of RNasin (Promega) and 20 units of RQI DNase (Promega) in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. After extraction with phenol/$CHCl_3$ (1:1) followed by $CHCl_3$, the supernatant was ethanol precipitated in the presence of 0.3 M NaOAC and RNA was redissolved in 0.1 × Tris-EDTA in diethyl pyrocarbonate-treated water. Reverse transcription was performed by incubating 1 μg of the DNase treated RNA with 300 units of Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT) (Gibco/BRL) in the presence of 15 μM oligo-dT (12–18) (Pharmacia) as a primer and 20 μM dNTP for 10 min at room temperature, 45 min at 42°C, 5 min at 99°C and 5 min at 5°C in the Gene Amp PCR system 9600 (Perkin Elmer Cetus, San Diego, CA). One half of the reaction was subsequently used for 30 cycles of PCR amplifications using GeneAmp PCR reagent kit (Perkin Elmer Cetus). PCR cycles include denaturation for 40 s at 94°C, annealing for 1 min at 61°C and polymerization for 1 min at 72°C. A minimum of three independent PCR amplifications from each specimen, for each experiment, were performed to guard against potential errors due to Taq polymerase misincorporation.

Oligonucleotides, cloning and sequencing of RT-PCR products

A pair of primers L1CYCE: 5′-GGGATGCGAAGGA-GCGGGACA-3′ and R1CYCE: 5′-AGCGGCGCAAC-TGTCTTTGGT-3′ based on the mRNA sequence of cyclin E (Koff *et al*., 1991; Lew *et al*., 1991) were designed to amplify the entire cyclin E coding sequence of human cyclin E cDNA (1250 bp, i.e. from nucleotide −23 to + 1227). To specifically amplify the cyclin E transcripts harboring the Δ9 and Δ148 deletion, the following sets of primers were used respectively: LMEMARK3: 5′-GC-AAACGTGACCGTTG-3′ and R1CYCE: 5′-AGCGGCG-CAACTGTCTTTGGT-3′; L1CYCE: 5′-GGGATGCGA-AGGAGCGGGACA-3′ and RMEMARK3: 5′-ACCG-CTCTGTGCTTCATC-3′. The PCR products were visualized by fractionating 1/5th of each reaction on a 1.5% agarose gel stained with ethidium bromide. A fraction of each reaction was then used to clone the RT-PCR products into the PCR II vector using the TA cloning system from Invitrogen (San Diego, CA). Plasmid DNA sequencing of cloned cDNA products with either T7 of SP6 primer was carried out using Sequenase 2.0 sequencing kit from United States Biochemicals Co (Cleveland, OH). Fifteen clones from each independent RT-PCR reaction (at least three) were completely sequenced in both orientations to confirm the sequences for Δ9 and Δ148 variants of cyclin E.

In vitro *translation*

To transcribe and translate the cyclin E cDNAs cloned in the PCR II vector we used the TNT coupled Reticulocyte Lysate system (Promega). Briefly, 1 μg of PCR II vector containing either cyclin E-wt, cyclin E-Δ9 or cyclin E-Δ148 was added to rabbit reticulocyte lysate (50% of total volume) in the presence of T7 RNA polymerase, 1 mM amino acid mixture minus methionine, 40 μCi [^{35}S]methionine (Dupont), RNasin ribonuclease inhibitor and the TNT reaction buffer (Promega) in a total volume of 50 μl. For non-radioactive reactions, unlabeled methionine was added to the mix and radioactivity was excluded. The reactions were then incubated at 30°C for 2 h and the translated radioactive products were separated by SDS–PAGE. The gels were then stained, destained, fluorographed, dried and the protein products were visualized by autoradiography. For visualization of the non-radioactive samples, the translation products were subjected to Western blot analysis using a polyclonal antibody specific to cyclin E.

Production of cyclins and kinases in insect cells

The cDNAs of cyclin E wild type, cyclin E-Δ9, cyclin E-Δ148 and cdk2 were subcloned into pMP3 (Pharmingen) plasmid containing the Basic Protein promoter that is active during and after viral DNA synthesis when the cell is producing baculovirus components to assemble the virus particles. At this stage there are larger numbers of modifying enzymes present which will increase the effectiveness of post-translational modification of the gene product of interest. Once the plasmids were constructed, they were individually co-transfected in sf9 insect cells with the linearized BaculoGold (Pharmingen) virus DNA (containing a lethal deletion), which through recombination would only produce viable recombinant baculovirus expressing our clones. The titer of all the supernatants were determined and insect cells were then infected with a plaque-forming unit/cell number of 1.

Acknowledgements

The authors wish to thank Drs Julie Gray-Bablin, Michael Koonce, Dan Rosen, Jim Dias and John Galivan for the critical reading of this manuscript; Dr Andy Koff for helpful discussions during the course of this work; Drs L-H Tsai and E Harlow for polyclonal antibody to cdk2; Dr E Lees for monoclonal (HE12) antibody to cyclin E; Drs J Roberts and A Koff for polyclonal antibody to cyclin E; and Dr W Harper for polyclonal antibody to cyclin A. We are grateful to Dr R Sager for providing cell lines and Dr AB Pardee, in whose lab some of these experiments were performed. We thank Dr Gyongyi Molnar and Ms Nuala O'Leary for excellent technical assistance. We also gratefully acknowledge the use of Wadsworth Center's Immunology, Molecular Biology, Photography/Graphics and Tissue Culture core facilities. This research was supported in part by Grant No. DAMD-17-94-J-4081, AIBS no. 1579 from the US Army Medical Research Acquisition Activity to KK.

References

Band V and Sager R. (1989). *Proc. Natl. Acad. Sci. USA*, **86,** 1249–1253.
Bianchi AB, *et al.* (1993). *Oncogene*, **8,** 1127–1133.
Buckley MF, *et al.* (1993). *Oncogene*, **8,** 2127–2133.
Crissman HA and Tobey RA. (1974). *Science*, **184,** 1287–1298.
Draetta GF. (1994). *Curr. Opin. Cell Biol.*, **6,** 842–846.
Elledge SJ and Spottswood MR. (1991). *EMBO J.*, **10,** 2643–2659.
Evans T, *et al.* (1983). *Cell*, **33,** 389–396.
Heichman, KA and Roberts JM. (1994). *Cell*, **79,** 557–562.
Hinds PW, *et al.* (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 709–713.
Hinds PW, *et al.* (1992). *Cell*, **70,** 993–1006.
Hunter T and Pines J. (1991). *Cell*, **66,** 1071–1074.
Hunter T and Pines J. (1994). *Cell*, **79,** 573–582.
Jiang W, *et al.* (1992). *Cancer Res.*, **52,** 2980–2983.
Jiang W, *et al.* (1993a). *Oncogene*, **8,** 3447–3457.
Jiang W, *et al.* (1993b). *Proc. Natl. Acad. Sci. USA*, **90,** 9026–9030.
Keyomarsi K, *et al.* (1994). *Cancer Res.*, **54,** 380–385.

Keyomarsi K and Pardee AB. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 1112–1116.
Keyomarsi K, Sandoval L, Band V and Pardee AB. (1991). *Cancer Res.*, **51,** 3602–3609.
King RW, Jackson PK and Kirschner MW. (1994). *Cell*, **79,** 563–571.
Koff A, *et al.* (1991). *Cell*, **66,** 1217–1228.
Koff A, *et al.* (1992). *Science*, **257,** 1689–1694.
Lammie GA, *et al.* (1991). *Oncogene*, **6,** 439–444.
Leach SF, *et al.* (1993). *Cancer Res.*, **53,** 1986–1989.
Lees E, *et al.* (1992). *Genes Dev.*, **6,** 1874–1855.
Lew DJ, Dulic V and Reed SI. (1991). *Cell*, **66,** 1197–1206.
Lovec H, *et al.* (1994). *Oncogene*, **9,** 323–326.
Matsushime H, Roussel MF, Ashman RA and Sherr CJ. (1991). *Cell*, **65,** 701–713.
Morgan DO. (1995). *Nature*, **374,** 131–134.
Motokura T and Arnold A. (1993). *Curr. Opin. Genet. & Devel.*, **3,** 5–10.
Motokura T, *et al.* (1991). *Nature*, **350,** 512–515.
Musgrove EA, Lee CSL, Buckley MF and Sutherland RL. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 8022–8026.
Nurse P. (1994). *Cell*, **79,** 547–550.
Ohtsubo M and Roberts JM. (1993). *Science*, **259,** 1908–1912.
Ohtsubo M, *et al.* (1995). *Mol. Cell. Biol.*, **15,** 2612–2624.
Quelle DE, *et al.* (1993). *Genes & Dev.*, **7,** 1559–1571.
Rao PN and Johnson RT. (1970). *Nature*, **225,** 159–164.
Resnitzky D, Gossen M, Bujard H and Reed SI. (1994). *Mol. Cell. Biol.*, **14,** 1669–1679.
Rosenberg CL, *et al.* (1991a). *Oncogene*, **6,** 449–453.
Rosenberg CL, *et al.* (1991b). *Proc. Natl. Acad. Sci. USA*, **88,** 9638–9642.
Rosenwald IB, Lazaris-Karatzas A, Sonenberg N and Schmidt EV. (1993). *Mol. Cell. Biol.*, **13,** 7358–7363.
Sewing A, *et al.* (1994). *J. Cell Sci.*, **107,** 581–588.
Sherr CJ. (1993). *Cell*, **73,** 1059–1065.
Sherr CJ. (1994). *Cell*, **79,** 551–555.
Soule HD, *et al.* (1990). *Cancer Research*, **50,** 6075–6086.
Standart N, Minshull J, Pines J and Hunt T. (1987). *Dev. Biol.*, **124,** 248–258.
Swenson KI, Farrell KM and Ruderman JV. (1986). *Cell*, **47,** 861–870.
Wang TC, *et al.* (1994). *Nature*, **369,** 669–671.
Withers D, *et al.* (1991). *Mol. Cell Biol.*, **11,** 4846–4853.
Xiong Y, Connolly T, Futcher B and Beach D. (1991). *Cell*, **65,** 691–699.